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Study of methanogenic enrichment cultures of rock cores from the deep subsurface of the Iberian Pyritic Belt

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Abstract

Two deep boreholes were drilled at 320 and 620 meters below surface in the Iberian Pyritic Belt (IPB) at Peña de Hierro (Huelva, Southwestern Spain). Cores were sampled and used for the establishment of enrichment cultures with methanogenic activity. The cultivable diversity of these enrichments was accessed using different cultivation techniques and several isolates were recovered in pure culture from various depths in both boreholes. Although no archaeal isolates were obtained in pure culture, strict anaerobes and facultative anaerobic bacteria belonging to the phyla *Proteobacteria, Firmicutes*, *Actinobacteria* and *Bacteroidetes* were isolated and identified using the 16S rRNA gene sequence.

Analysis of three selected enrichment cultures by amplification of both bacterial and archaeal 16S rRNA gene followed by pyrosequencing revealed further information on the populations enriched. The archaeal sequences obtained from the methanogenic enrichment cultures belonged to the orders *Methanosarcinales* and

Methanocellales. To best of our knowledge this is the first report of enrichment in members of the *Methanocellales* in a deep terrestrial subsurface ecosystem. Several bacterial populations, predominantly consisting of *Firmicutes* and *Proteobacteria*, were also enriched. The prevalent microbial populations enriched as detected by pyrosequencing analysis, as well as the bacterial isolates cultivated were affiliated with known fermentative, sulfate reducing and acetogenic bacteria or methanogenic archaea. Our results show a great diversity in the microbial communities of the IPB deep subsurface.

Keywords: Microbiology, Earth sciences

1. Introduction

Microbial ecology study of deep subsurface environments is a fascinating field of research. Deep subsurface terrestrial environments have been studied at several locations around the world (Kotelnikova and Pedersen, 1998; Chivian et al., 2008; Fukuda et al., 2010; Hallbeck and Pedersen, 2012; Magnabosco et al., 2016), reporting that life is ubiquitous and can in fact extend several kilometers deep into the subsurface. While in the last decades its being increasingly recognized the importance of studying diversity and activities of deep subsurface communities, due to difficulties in accessing and sampling these environments, the deep subsurface still remains one of the least understood environments on Earth.

The deep terrestrial subsurface is characterized as an anaerobic, oligotrophic environment, where in the absence of light, and therefore in the absence of the direct influence from photosynthetic primary production, chemolithoautotrophic microorganisms (e.g. methanogens, acetogens) are thought to play an important role as primary producers (Pedersen, 1997; Stevens, 1997). As such, the study of subsurface environments represents as well, an important model for life on early Earth and as it may also occur in other planetary bodies.

The Iberian Pyritic Belt (IPB) is a geological formation that extends between south of Spain and Portugal, and constitutes one of the largest sulfide ore deposits known, yet the geomicrobiology of this subsurface environment is poorly characterized. Located in the south of Spain, Río Tinto has its origin at the core of the IPB in Peña de Hierro. Río Tinto is a known terrestrial analogue for some locations on Mars due to similar geochemical and mineralogical features (e.g. similarities between main sulfide bioleaching products on Río Tinto basin and sulfate and iron oxide deposits on Mars) (Amils et al., 2014).

Studies on the deep subsurface of the IPB at Peña de Hierro began with Mars Astrobiology Research and Technology Experiment − MARTE project (2003−2006), which showed the presence of various substrates such as ferrous and ferric iron,

nitrate, nitrite, sulfate, methane, hydrogen and carbon dioxide that could support different anaerobic chemolithotrophic metabolisms in the subsurface. Catalyzed Reporter Deposition in situ Hybridization (CARD-FISH) studies and enrichment cultures also indicated the presence of viable and active microorganisms in the deep subsurface of the IPB (Amils et al., 2008; Fernández-Remolar et al., 2008; Puente-Sánchez et al., 2014).

A second drilling campaign (2011−2012) was done in the framework of the Iberian Pyrite Belt Subsurface Life project (IPBSL) which had as its main goal to further characterize the microbial diversity existing in the deep subsurface of the IPB and elucidate the interactions between microbial activities and the local geology. In this campaign two deeper boreholes were drilled, BH11 (320 m) and BH10 (620 m). Similarly to the previous study, analysis of rock samples indicated the presence of gases such as hydrogen, carbon dioxide and methane as well as anions such as the nitrate, nitrite, acetate, formate and propionate were detected within BH10 and BH11 boreholes (Amils et al., 2013). To address microbial diversity and establish a real understanding of the microbial community interaction with the local geology, rock core samples from 39 depths on BH10 and 20 depths on BH11 were sampled under anaerobic and sterile conditions.

The detection of known methanogenic substrates such as acetate, hydrogen and carbon dioxide as well as the detection of methane, the product of activity of methanogenic microorganisms, lead to the hypothesis that methanogenesis may comprise an important metabolism in the deep subsurface of the IPB. The objective of this study was to demonstrate the presence and identify the lineages of methanogens in the deep subsurface of the IPB. In addition, we aimed to isolate new deep subsurface microorganisms in pure culture.

Here, we report the application of culture dependent techniques for the establishment of enrichment cultures with rock samples from BH10 and BH11 boreholes, which successfully developed active methane production, indicating that methanogenic microorganisms occur in the subsurface of the IPB. Additionally, isolation of microorganisms from these enrichment cultures and identification by pyrosequencing analysis of both bacterial and archaeal 16S rRNA gene sequences, allowed the description of the microbial diversity enriched.

2. Materials and methods

2.1. Site description, borehole drilling and sampling

The IPBSL campaign at Peña de Hierro resulted in the drilling of two boreholes, BH11 and BH10, reaching a depth of 320 and 620 m, respectively. A chemical tracer (NaBr) was introduced in the drilling fluid to evaluate possible contamination of

samples during drilling. Cores were retrieved encased in plastic liners, placed in plastic bags and flushed with nitrogen to maintain anaerobic conditions, followed by transport to a nearby laboratory at the Museo Minero at Minas de Ríotinto for rock sampling in an anaerobic chamber. Core samples of selected sections were retrieved in sterile and anaerobic conditions using a hydraulic core splitter and a rotary hammer to remove the central part of the cores (Fig. 1). Temperature was measured with an infra-red thermometer to control temperature of the hammer bit. A total of 20 depths for BH11 and 39 depths for BH10 were sampled and used for preparation of enrichment cultures.

2.2. Methanogenic enrichment cultures

Rock samples ($\sim$6 g) corresponded to different depths along both boreholes were used as inoculum. All procedures were performed in an anaerobic chamber. For enrichment of methanogenic microorganisms, serum bottles with three different anoxic media were prepared, composed of the same basal mineral solution and differing in added energy sources. The basal media had the following composition (per liter of distilled water): 0.3 g NH_4Cl, 0.3 g $K_2HPO_4.3H_2O$, 0.1 g $MgSO_4.7H_2O$, 0.4 g $NaHCO_3$, 0.01 g $CaCl_2.2H_2O$, 0.1 g yeast extract, 1 ml trace element solution (Sanz et al., 1997) at an initial pH of 7. Cysteine (0.5 g/L) was used as reducing agent and resazurin (0.2 mg/L) was used as redox indicator. Medium designated MG1 was supplemented with 20 mM acetate and a headspace composed of $N_2:CO_2$ (80:20, v/v), medium MG2 with the gas mixture $H_2:CO_2$ (80:20, v/v) and medium MG3 was supplemented with a solution composed of propionate:butyrate:methanol (5 mM

Fig. 1. Drilling campaign at Peña de Hierro (a); core retrieved from the deep subsurface (b), recovering samples from the central area of the core inside an anaerobiose chamber (c, d); bottles with enrichment cultures used in this study (e); colonies on the surface of agar on roll tubes (f).

each) and a headspace composed of $N_2:CO_2$ (80:20, v/v). All supplements were added from anaerobic and sterile stock solutions. Enrichments were incubated at 30 °C without shaking for up to one year. Activity of the different cultures was measured by generation of methane at different intervals. After one year incubation, some of the bottles with positive activity for methanogenesis were chosen for isolation of enriched microorganisms.

2.3. Culturing techniques and media for isolation

Isolation of anaerobic microorganisms was performed by the Hungate roll-tube method (Hungate, 1969; Balch et al., 1979). Culture media for isolation of anaerobes had the same composition as the enrichment culture medium, including the addition of Agar Noble (15 g/L) (Difco) as solidifying agent. Hungate rolling tubes were inoculated with serial dilutions of each enrichment culture, respectively. Incubation was at 30 °C in the dark until development of isolated colonies. Colonies were picked in anaerobic conditions with a bent Pasteur pipette and transferred into culture plates with the same cultivation conditions. Plates were incubated in anaerobic jars with AnaeroGen sachets (Oxoid) to generate anaerobic conditions and in the case of cultures incubated with $H_2:CO_2$ (80:20, v/v), the gas mixture was injected inside the jar. Sub-culturing was done for at least two to three transfers. Colonies were screened for unique morphologies and representatives of each morphotype were selected for further identification.

2.4. Analytical measurement

Methanogenic activity was monitored by measurement of headspace methane levels by gas chromatography using a Varian Star 3400CX gas chromatographer equipped with a split/splitless injector and a flame ionization detector. Development of methane production over time was considered a positive indication for methanogenic activity.

2.5. Characterization of isolates

DNA was extracted using a cetyltrimethylammonium bromide (CTAB) based extraction method as described before (Wilson, 2001). The 16S rRNA gene was amplified by PCR using the primers 27F-1492R (Lane, 1991). PCR amplicons were generated with AmpliTaq DNA polymerase (Applied Biosystems) according to manufacturer instructions. The PCR conditions were the following: initial denaturation at 94 °C for 5 min followed by 30 cycles consisting of denaturation at 94 °C for 1 min, primer annealing for 1 min at 56 °C and elongation at 72 °C for 2 min. The final elongation was at 72 °C for 7 min. PCR products were checked for the correct length by electrophoresis on a 1% agarose gel stained with EtBr

and purified with a DNA purification JetQuick kit (Genomed). Purified DNA products were quantified using a Nanodrop ND-1000 device (Nanodrop, DE, USA) and sent for sequencing. Taxonomic identification of amplified sequences was assessed using EzTaxon-e (Kim et al., 2012).

2.6. Physiological tests

For some selected isolated strains, growth was tested in oligotrophic conditions both under anaerobic and aerobic conditions in basal media and in richer media under aerobic conditions. The oligotrophic media had the following composition (per liter of distilled water): 0.3 g NH_4Cl, 0.3 g $K_2HPO_4.3H_2O$, 0.1 g $MgSO_4.7H_2O$, 0.4 g $NaHCO_3$, 0.01 g $CaCl_2.2H_2O$, 0.1 g yeast extract, 10 ml trace element solution (DSMZ 141 medium) and 15 g Agar Noble (Difco). For the anaerobic media cysteine (0.5 g/L) was used to reduce the media and resazurin (0.1 mg/L) was added as a redox indicator. Anaerobic oligotrophic plates were incubated in anaerobic jars as previously described. Growth under aerobic conditions was also examined in the following media: R2A (Difco), Nutrient Agar (NA) (Difco), Tryptic Soy Agar (TSA) (Difco). All plates were incubated at 30 °C for up to 10 days.

2.7. Characterization of enrichment cultures

Three enrichment cultures, with positive methane production were chosen for DNA extraction followed by amplification of 16S rRNA gene and pyrosequencing analysis. The cultures selected were the following: T1.2MG culture (core sample from 63.55 meters below surface (mbs), BH11) enriched with acetate under an atmosphere of $N_2:CO_2$ (80:20, v/v), T2.22MG culture (core sample from 450.3 mbs, BH10) enriched with a mix of propionate:methanol:butyrate under an atmosphere of $N_2:CO_2$ (80:20, v/v) and T2.26MG culture (core sample from 492.6 mbs, BH10) enriched with $H_2:CO_2$ (80:20, v/v).

Total DNA from the enrichment cultures was extracted with PowerSoil DNA extraction kit (MoBio Laboratories Inc., CA). Culture samples were centrifuged to recover cell pellets and manufacturer's instructions were followed for DNA extraction from cell pellets. Archaeal and bacterial amplification libraries for high throughput 454 pyrosequencing were prepared by PCR amplification of partial 16S rRNA gene sequence using Invitrogen PlatinumTaq DNA polymerase enzyme, following manufacturer instructions. Bacterial 16S rRNA gene fragments were amplified with the primer pair 27F-907R (Lane, 1991). Archaeal 16S rRNA gene fragments were amplified with primer pair 21F-915R (Stahl and Amann, 1991; DeLong, 1992). PCR conditions were the following: initial denaturation at 95 °C for 3 min followed by 28 cycles consisting of denaturation at 95 °C for 30 sec, primer annealing for 45 sec at a 54 °C and elongation at 68 °C for 1.30 min. The final elongation was at 68 °C for 10 min. Correct size of the PCR products was checked with agarose gel

electrophoresis in a EtBr (0.05 μg/ml final concentration) stained 1% agarose gel in 1% Tris-Acetate-EDTA (TAE) buffer. PCR amplification products were purified with the Invitrogen Purelink kit. Pyrosequencing was performed by Centro de Investigación Tecnología y Innovación (CITIUS, University of Sevilla, Spain) using a 454 FLX + System (Roche).

All sequence processing analysis was done using the software Mothur v.1.36.1 (Schloss et al., 2009). Sequences were trimmed to remove primers and barcodes. Sequences with more than eight homopolymers and with ambiguous bases were also excluded. Further quality filtering of the resulting sequences dependent on base quality scores was performed using the script moira.py (https://github.com/fpusan/moira), which contains the python implementation of the Poisson binominal filtering algorithm (Puente-Sánchez et al., 2016). In addition, sequences with a length of less than 400 bp (for bacteria) and 600 bp (for archaea) were excluded. The sequences obtained after the quality filtering were aligned against the SILVA 16S rRNA reference alignment v123 (Pruesse et al., 2007). A further pre-clustering step was performed by clustering reads that differed in 1 base. The UCHIME algorithm as implemented in Mothur, was used to detect chimeras, which were then removed from the dataset. Pairwise distance matrices were built and the sequences were clustered into OTU's at 97% similarity. Taxonomic classification was performed with the SILVA 16S rRNA gene database v123 (with a 80% confidence threshold). Rarefaction curves were calculated for each sample.

2.8. Nucleotide sequence accession number

The 16S rRNA bacterial gene sequences obtained in this study have been deposited at the DDBJ/ENA/GenBank under the accession numbers [MF361863−MF361869, MF361871, MF361873−MF361879; MF361881, MF361882, MF361883, MF361885, MF361886, MF361888, MF361889, MF361890, MF361893, MF361894, MF361895]. The raw sequences from pyrosequencing obtained in this study were deposited in the National Center for Biotechnology Information (NCBI) under accession numbers SRR6186122 − SRR6186127.

3. Results

Enrichment cultures showed positive activity at several depths, throughout the borehole. Enrichments with methane production of at least 10 times higher than that of the control without added substrate were selected for isolation studies (Table 1).

Screening of colonies for unique morphologies led to the selection of representative isolates of each morphotype for further identification based on 16S rRNA gene. All isolates belonged to the domain *Bacteria* and grouped within the phyla *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes* (Table 2). The majority of the

Table 1. Summary of the enrichment cultures selected with methanogenic activity, respective depths of rock cores (meters below surface in **bold**) and substrate used to promote growth.

Borehole	Enrichment	Substrate added
BH11	*T1.2MG_**63.55**	Acetate, N_2:CO_2
BH10	T2.5_**139.5**	H_2:CO_2
	T2.6_**206.6**	H_2:CO_2
	T2.7_**228**	H_2:CO_2
	T2.8_**249.8**	H_2:CO_2
	T2.9_**266.3**	H_2:CO_2
	T2.10_**284**	H_2:CO_2
	T2.11_**284**	H_2:CO_2
	*T2.22MG_**450.3**	Mix propionate:methanol:butyrate, N_2:CO_2
	*T2.26MG_**492.6**	H_2:CO_2
	T2.31MG_**414**	Acetate, N_2:CO_2

[*] Cultures selected for characterization of the enriched community by 16S rRNA pyrosequencing analysis.

bacterial isolates belonged to *Proteobacteria* and *Actinobacteria* (Table 2). Within the phylum *Actinobacteria* strains belonging to the following genera were isolated: *Tessaracoccus* (isolated with H_2/CO_2 from 139.4, 206.6 and 284 mbs, BH10), *Microbacterium* (isolated with H_2/CO_2 from 284 mbs, BH10), *Cellulomonas* (isolated with a mix of propionate/butyrate/methanol plus N_2/CO_2 from 450.3 mbs and with acetate plus N_2/CO_2 from 414 mbs, BH10), *Aestuariimicrobium* (isolated with H_2/CO_2 from 492.6 mbs, BH10) and, *Nocardioides* (isolated with H_2/CO_2 from 492.6 mbs, BH10); in the phylum *Proteobacteria* strains related to the genera *Pleomorphomonas* (isolated with acetate plus N_2/CO_2 from 63.55 mbs, BH11), *Pseudomonas* (isolated with H_2/CO_2 from 139.4, 206.6 and 492.6 mbs, BH10), *Rhodoplanes* (isolated with H_2/CO_2 from 284 and 492.6 mbs and with acetate plus N_2/CO_2 from 414 mbs, BH10), *Desulfovibrio* (isolated with a mix of propionate/butyrate/methanol plus N_2/CO_2 from 450.3 mbs, BH10), *Brevundimonas* (isolated with H_2/CO_2 from 492.6 mbs, BH10) and *Rhizobium* (isolated with H_2/CO_2 from 492.6 mbs, BH10); in the phylum *Firmicutes* isolates belonging to the genera *Paenibacillus* (isolated with H_2/CO_2 from 139.4 mbs, BH10), *Bacillus* (isolated with H_2/CO_2 from 266.3 mbs, BH10), and *Acetoanaerobium* (isolated with a mix of propionate/butyrate/methanol plus N_2/CO_2 from 450.3 mbs, BH10); and in the phylum *Bacteroidetes* strains affiliated to the genera *Macellibacteroides* (isolated with a mix of propionate/butyrate/methanol plus N_2/CO_2 from 450.3 mbs, BH10 and with acetate plus N_2/CO_2 from 63.55 mbs, BH11) and *Propionicimonas* (isolated with acetate plus N_2/CO_2 from 414 mbs, BH10).

No *Archaea* isolates were obtained in pure culture under the culture conditions used. Enrichment cultures T2.7 and T2.8 failed to produce any isolates. Furthermore, isolates related to *Macellibacteroides* sp. failed to grow again after a few transfers.

Table 2. Summary of depth of rock cores where microorganisms were isolated from, isolation conditions and taxonomic affiliation of each isolate based on the sequencing of the 16S rRNA gene.

Borehole	Depth (m)	Strains	Isolation media	Phylum	Genera	Closest strain in EzTaxon-e database (% 16S rRNA similarity)	Putative designation of the isolated representative strain(s)
BH11	63.55	T1.2MG-59	Basal media + acetate + $N_2:CO_2$	*Bacteriodetes*	*Macellibacteroides*	*Macellibacteroides fermentans* LIND7H[T] (99.56)	*Macellibacteroides* sp. T1.2MG-59
		T1.2MG-36	Basal media + acetate + $N_2:CO_2$	*Proteobacteria*	*Pleomorphomonas*	*Pleomorphomonas oryzae* DSM 16300[T] (99.70)	*Pleomorphomonas* sp. T1.2MG-36
BH10	139.4	T2.5-30	Basal media + $H_2:CO_2$	*Actinobacteria*	*Tessaracoccus*	*Tessaracoccus lapidicaptus* IPBSL-7[T] (100)	*Tessaracoccus* sp. T2.5-30
		T2.5-50		*Actinobacteria*	*Tessaracoccus*	*Tessaracoccus oleiagri* SL014B-20A1(T) (99.85)	*Tessaracoccus* sp. T2.5-50
		T2.5-46A		*Firmicutes*	*Paenibacillus*	*Paenibacillus odorifer* DSM 15391[T] (98.84)	*Paenibacillus* sp. T2.5-46A
		T2.5-7.3		*Proteobacteria*	*Pseudomonas*	*Pseudomonas songnenensis* NEAU-ST5-5[T] (99.22)	*Pseudomonas* sp. T2.5-7.3
	206.6	T2.6-4D2	Basal media + $H_2:CO_2$	*Actinobacteria*	*Tessaracoccus*	*Tessaracoccus lapidicaptus* IPBSL-7[T] (100)	*Tessaracoccus* sp. T2.6-4D2
		T2.6-12		*Proteobacteria*	*Pseudomonas*	*Pseudomonas songnenensis* NEAU-ST5-5[T] (99.85)	*Pseudomonas* sp. T2.6-12
	266.3	T2.9-1	Basal media + $H_2:CO_2$	*Firmicutes*	*Bacillus*	*Bacillus circulans* ATCC 4513[T] (98.98)	*Bacillus* sp. T2.9-1
	284	T2.10-17	Basal media + $H_2:CO_2$	*Actinobacteria*	*Tessaracoccus*	*Tessaracoccus lapidicaptus* IPBSL-7[T] (100)	*Tessaracoccus* sp. T2.10-17
		T2.11-28			*Microbacterium*	*Microbacterium saccharophilum* K-1[T] (99.42)	*Microbacterium* sp. T2.11-28
		T2.11-4.1		*Proteobacteria*	*Rhodoplanes*	*Rhodoplanes piscinae* JA266[T] (99.85)	*Rhodoplanes* sp. T2.11-4.1
	414	T2.31MG-40	Basal media + acetate + $N_2:CO_2$	*Actinobacteria*	*Cellulomonas*	*Cellulomonas hominis* DMMZ CE40[T] (98.88)	*Cellulomonas* sp. T2.31MG-40
		T2.31MG-18	Basal media + acetate + $N_2:CO_2$			*Cellulomonas pakistanensis* NCCP-11[T] (97.66)	*Cellulomonas* sp. T2.31MG-18
		T2.31MG-1	Basal media + acetate + $N_2:CO_2$	*Bacteriodetes*	*Propionicimonas*	*Propionicimonas paludicola* DSM 15597[T] (98.53)	*Propionicimonas* sp. T2.31MG-1
		T2.31MG-Q8	Basal media + acetate + $N_2:CO_2$	*Proteobacteria*	*Rhodoplanes*	*Rhodoplanes piscinae* JA266[T] (99.85)	*Rhodoplanes* sp. T2.31MG-Q8

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Heliyon

Article No~e00605

Table 2. (*Continued*)

Borehole	Depth (m)	Strains	Isolation media	Phylum	Genera	Closest strain in EzTaxon-e database (% 16S rRNA similarity)	Putative designation of the isolated representative strain(s)
	450.3	T2.22MG-43	Basal media + propionate: methanol:butyrate+ N$_2$:CO$_2$	*Actinobacteria*	*Cellulomonas*	*Cellulomonas fimi* ATCC 484[T] (97.65)	*Cellulomonas* sp. T2.22MG-43
		T2.22MG-48A		*Bacteriodetes*	*Macellibacteroides*	*Macellibacteroides fermentans* LIND7H[T] (99.56)	*Macellibacteroides* sp. T2.22MG-48A
		T2.22MG-11		*Firmicutes*	*Acetoanaerobium*	*Acetoanaerobium noterae* ATCC 35199[T] (98.41)	*Acetoanaerobium* sp. T2.22MG-11
		T2.22MG-35		*Proteobacteria*	*Desulfovibrio*	*Desulfovibrio oxamicus* DSM 1925[T] (99.14)	*Desulfovibrio* sp. T2.22MG-35
	492.6	T2.26MG-19.2B	Basal media + H$_2$:CO$_2$	*Actinobacteria*	*Aestuariimicrobium*	*Aestuariimicrobium kwangyangense* R27[T] (99.93)	*Aestuariimicrobium* sp. T2.26MG-19.2B
		T2.26MG-1			*Nocardiodes*	*Nocardioides pyridinolyticus* OS4(T) (97.90)	*Nocardiodes* sp. T2.26MG-1
		T2.26MG-97		*Proteobacteria*	*Brevundimonas*	*Brevundimonas mediterranea* V4.BO.10[T] (99.77)	*Brevundimonas* sp. T2.26MG-97
		T2.26MG-48.2			*Pseudomonas*	*Pseudomonas stutzeri* ATCC 17588[T] (99.93)	*Pseudomonas* sp. T2.26MG-48.2
		T2.26MG-10			*Rhizobium*	*Rhizobium naphthalenivorans* TSY03b[T] (98.88)	*Rhizobium* sp. T2.26MG-10
		T2.26MG-112.2					*Rhizobium* sp. T2.26MG-112.2
		T2.26MG-98			*Rhodoplanes*	*Rhodoplanes piscinae* JA266[T] (100)	*Rhodoplanes* sp. T2.26MG-98

For some strains, growth in anaerobic and oligotrophic conditions was also tested to evaluate the effect of the agar matrix on growth. Although weak, the majority of the tested strains could grow in these conditions (Table 3). Capability to grow in aerobic conditions in oligotrophic media as well as richer media was also verified (Table 3).

Pyrosequencing of bacterial and archaeal 16S rRNA gene sequences from three selected rock core enrichment cultures with active methane production (Table 1), produced a total of 29686 bacterial and 19039 archaeal high quality reads for culture T1.2MG; 28589 bacterial and 3321 archaeal high quality reads for culture T2.22MG; and 12853 bacterial and 24007 archaeal high quality reads for culture T2.26MG. Rarefaction analysis of enriched bacterial community showed no saturation in samples from both cultures T1.2MG and T2.26MG, while it reached saturation for the samples from culture T2.22MG (Fig. 2a). In the case of rarefaction analysis of the archaeal community, samples from cultures T1.2MG and T2.26MG reached saturation while no saturation was reached for the sample from

Table 3. Growth of isolates on several media. +, positive; −, negative; w, weak; vw-very weak.

|	R2A (Aerobiosis)	TSA (Aerobiosis)	NA (Aerobiosis)	Oligotrophic media (Aerobiosis)	Oligotrophic media (anaerobiosis)
Aestuariimicrobium sp. T2.26MG-19.2B	+	+	+	w	w
Brevundimonas sp. T2.26MG-97	+	+	+	w	w
Microbacterium sp. T2.11-28	+	+	+	w	w
Nocardioides sp. T2.26MG-1	+	−	+	+	+
Paenibacillus sp. T2.5-46A	+	+	+	−	−
Rhizobium sp. T2.26MG-10	+	+	−	+	+
Rhodoplanes sp. T2.26MG-98	+	−	+	w	w
Tessaracoccus sp. T2.5-30	+	+	+	−	−
Tessaracoccus sp. T2.5-50	+	+	+	−	−
Pseudomonas sp. T2.26MG-48.2	+	+	+	w	w
Cellulomonas sp. T2.22MG-43	+	w	+	vw	vw
Pleomorphomonas sp. T1.2MG-36	+	+	+	vw	vw
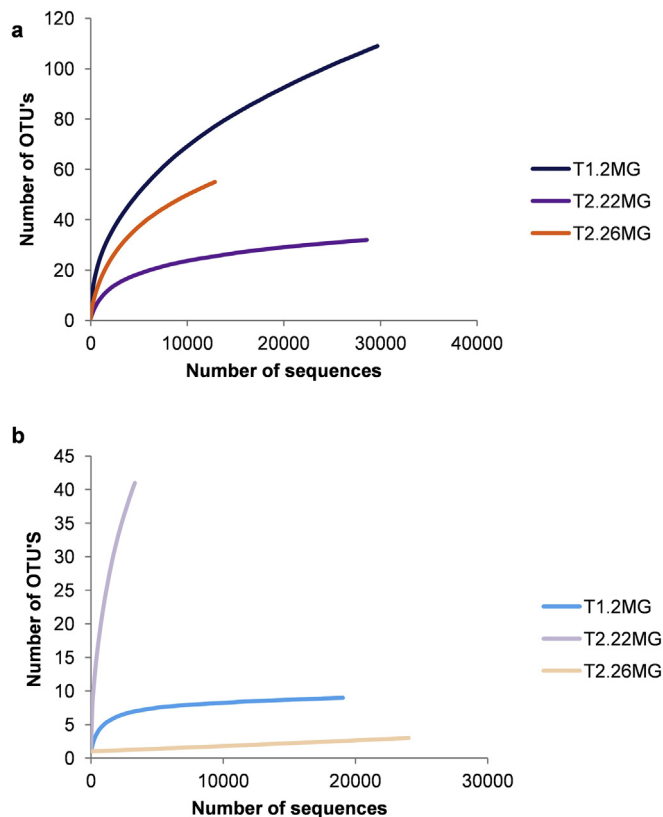
Fig. 2. Rarefaction curves for bacterial OTU's (97 % cutoff) (a) and rarefaction curves for archaeal OTU's (97 % cutoff) (b) from enrichment cultures T1.2MG, T2.22MG and T2.26MG.

T2.22MG culture (Fig. 2b). For the cases where saturation was not reached, samples may require further sequencing to ensure the entire diversity present was captured.

The majority of the identified OTU's in the three cultures at the phylum level are affiliated with *Proteobacteria* and *Firmicutes* (Fig. 3), with minor abundant OTU's ($\leq$0.3%) affiliated with the phyla *Bacteroidetes*, *Actinobacteria*, *Chloroflexi* or remained unclassified.

Culture T1.2MG (enriched with acetate plus N_2/CO_2) presented the most diverse bacterial populations. At the genus level (Fig. 4) the major enriched bacterial OTU's were affiliated with the genera *Sedimentibacter* (43.50%), *Erysipelothrix* (42.58%), and *Rhodoplanes* (5.50%). Other less abundant OTU's at the genus level were classified as *Desulfosporosinus* (2.22%) and *Sporacetigenium* (1.30%) or either assigned as unclassified (4.11%). In culture T2.22MG (enriched with a mix of propionate/methanol/butyrate plus N_2/CO_2), pyrosequencing analysis indicated that the most abundant bacterial group was closely related to the genus *Azospira* (99.85%) (Fig. 4). Less abundant OTU's enriched in this culture were classified as affiliated with *Syntrophomonas* (0.33%) or assigned as unclassified (0.45%). For culture T2.26MG (enriched with H_2/CO_2) the major enriched OTU's were closely related
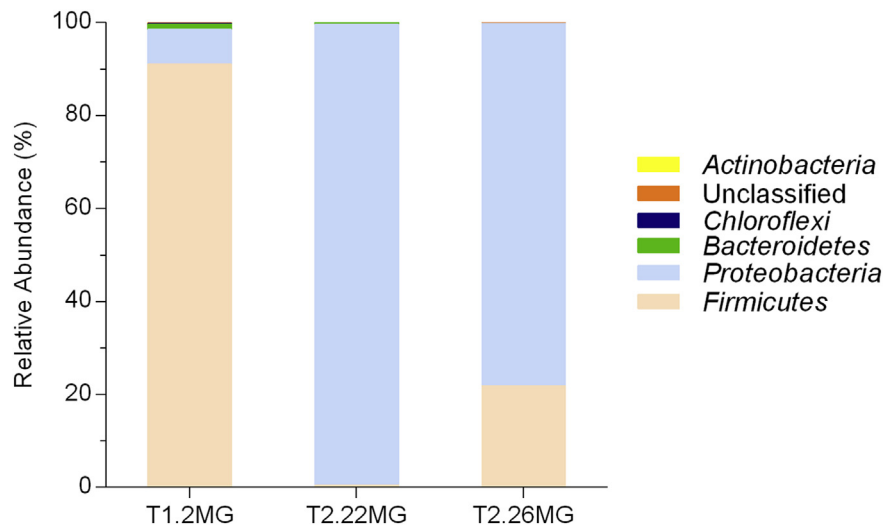
Fig. 3. Relative abundance of bacterial sequences at the phylum level for cultures T1.2MG, T2.22MG and T2.26MG. OTU's which could not be taxonomically assigned at the phylum level are reported as "Unclassified".
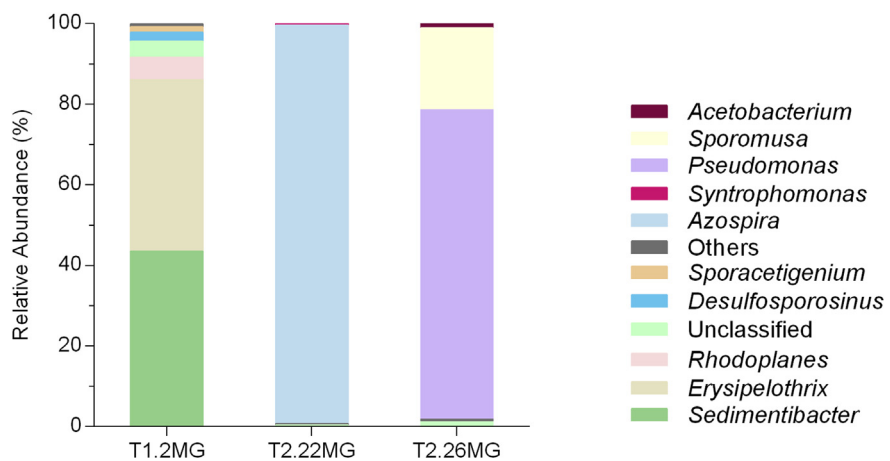
Fig. 4. Relative abundance of bacterial sequences at the genus level for cultures T1.2MG, T2.22MG and T2.26MG. Classified genera with relative abundances above the cutoff value of 0.3% are indicated. OTU's which could not be taxonomically assigned at the genus level are reported as "Unclassified". The category "Others" comprise all the genera with a relative abundance of <0.3%.

to the genus *Pseudomonas* (76.74%) and to the genus *Sporomusa* (20.28%) (Fig. 4). Other less abundant OTU's at the genus level were classified as related to the genera *Acetobacterium* (1.06%) or assigned as unclassified (1.22%).

In both T1.2MG and T2.26MG cultures the major enriched archaeal populations were affiliated at the genus level with *Methanosarcina*, which groups within the order *Methanosarcinales* on the *Euryarchaeota* phylum (Table 4). In culture T2.22MG, while only one of less abundant OTU's was classified at the genus level as *Methanosarcina* (12.89%), the major enriched OTU's were affiliated with the

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Table 4. Relative abundance of archaeal sequences for samples T1.2MG, T2.22MG and T2.26MG at the phylum, class, order, family and genus levels.

Sample	Phylum	Class	Order	Family	Genus
T1.2MG	*Euyarchaeota* (100%)	*Methanomicrobia* (100%)	*Methanosarcinales* (99.85%) Others (0.15%)	*Methanosarcinaceae* (99.85%) Others (0.15%)	*Methanosarcina* (99.84%) Others (0.16%)
T2.22MG	*Euyarchaeota* (100%)	*Methanomicrobia* (100%)	*Methanocellales* (86.72%) *Methanosarcinales* (12.89%) Unclassified (0.39%)	*Methanocellaceae* (86.72%) *Methanosarcinaceae* (12.89%) Unclassified (0.39%)	Rice Cluster I (46.55%) *Methanocella* (40.14%) *Methanosarcina* (12.89%) Unclassified (0.42%)
T2.26MG	*Euyarchaeota* (100%)	*Methanomicrobia* (100%)	*Methanosarcinales* (100%)	*Methanosarcinaceae* (100%)	*Methanosarcina* (100%)

Confidence levels higher than 80% have been considered. Percentages have been calculated considering the total of high quality reads for each samples, T1.2MG (19039), T2.22MG (3321) and T2.26MG (24007).

Rice Cluster I (46.55%) or with the *Methanocella* genus (40.14%) within the order *Methanocellales* on the *Euryarchaeota* phylum (Table 4).

4. Discussion

The establishment of several enrichment cultures supplemented with methanogenic substrates and inoculated with various rock cores, resulted in the successful development of methane production from various depths on both BH10 and BH11 boreholes and with all the substrate mixes tested. These results showed the presence of viable and active methanogenic populations within the sampled rocks. Gases such as methane, hydrogen and carbon dioxide, as well as, soluble inorganic anions such as nitrate, nitrite and organic acids as acetate, propionate or formate have been detected from different depths along both boreholes (Amils et al., 2014). Consequently, substrates able to sustain methanogenic activities were present in the system and could indeed be used to support in situ the growth of the methanogenic populations enriched in these cultures.

To identify the enriched microbial diversity, several enrichment cultures with active methane production (Table 1) were selected for isolation studies and three of these cultures, each one supplemented with a distinct substrate mix, were further chosen to investigate the enriched microbial diversity by pyrosequencing of the 16S rRNA gene.

Isolation and identification of the cultured diversity obtained using traditional culture techniques showed that all isolates belonged to the phyla *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes* within the domain *Bacteria* (Table 2). Interestingly, the majority of the isolates showed high similarity, based on 16S rRNA gene sequence analysis with cultured strains previously isolated from other habitats, such as marine environment, freshwater, soils, sediments, or wastewater treatment plants. Identification based on sequencing of the 16S rRNA gene demonstrated the isolation of bacterial strains affiliated with diverse genera as shown in Table 2. Isolates affiliated with the genera *Acetoanaerobium, Desulfovibrio* and *Macellibacteroides* are strict anaerobes, while the remaining organisms isolated under strict anaerobiosis (Table 2) could also grow under aerobic conditions in R2A medium. Growth of some of the facultative anaerobic isolates was also further tested in other richer media than R2A (Table 3), i.e. TSA media or NA media. All tested strains demonstrated capability to grow on rich media, with higher biomass production, similarly to what has been described for their closest phylogenetic relatives.

Pyrosequencing analysis of the bacterial 16S rRNA gene identified additional bacterial microbial diversity enriched in all the three studied cultures which were grown with a different set of substrates, i.e. with either acetate plus N_2/CO_2, a mixture of propionate/methanol/butyrate plus N_2/CO_2 or under an atmosphere of H_2/CO_2

(Figs. 3 and 4). Once more, it is observed a striking variability between the populations enriched under each condition. In culture T1.2MG (enriched with acetate plus N_2/CO_2, BH11, 63.55 mbs) bacteria affiliated with genera *Sedimentibacter, Erysipelothrix, Rhodoplanes, Desulfosporosinus* and *Sporacetigenium* were enriched. In culture T2.22MG (enriched with a mix of propionate/methanol/butyrate plus N_2/CO_2 in headspace, BH10, 450.3 mbs) were detected sequences affiliated with the genera *Azospira* and *Syntrophomonas* and finally in culture T2.26MG (enriched with H_2/CO_2, BH10, 492.6 mbs) bacteria affiliated with the genera *Pseudomonas, Sporomusa* and *Acetobacterium* were enriched.

The diversity of isolates recovered in culture (Table 2) and the microbial diversity enriched as detected by pyrosequencing analysis (Figs. 3 and 4), reflects not only the diverse growth conditions applied, i.e. the substrates supplied, but are as well a reflection of the diversity of microbial populations that inhabit rocks at the different depths sampled. The rock subsurface environment is a heterogeneous environment as a result of its geochemical and mineralogical variability. The porous and fractured nature of the rock matrix offers the possibility for life to thrive on microniches which may be connected or independent from each other, even at a short distance apart. This allows diverse microbial metabolisms to occur within the rock matrix. Each microniche, even if just a few millimeters apart can present different proprieties. The active metabolisms within Río Tinto subsurface aquifer will ultimately depend on the physicochemical characteristics (e.g. rock composition, energy and nutrient sources available, water availability, temperature, pressure, pore space) established within each microniche on the rock matrix.

Several bacterial isolates (Table 2) and bacterial 16S rRNA sequences (Figs. 3 and 4) were recovered from enrichment cultures, established under an atmosphere of hydrogen and carbon dioxide. For bacterial strains isolated under such conditions, further testing of growth in anaerobic conditions in the presence of just the basal solid media (Table 3) used for isolation, indicated that the tested strains can still grow, although weakly, in these conditions. This suggests that growth was supported by the trace quantities of yeast extract incorporated in the media as a source of growth factors, and also the presence of residual organic molecules in the agar used as solidifying agent may have played a role in support growth. Therefore, these strains demonstrate oligotrophic capabilities which are in accordance with their presence in an oligotrophic environment as is the deep subsurface.

This study is the first report from the IPB subsurface of the isolation of an acetogenic bacterial strain (*Acetoanaerobium* sp. T2.22MG-11, Table 2) as well as the identification of 16S rRNA sequences affiliated with known acetogenic bacteria such as members of the genera *Sporomusa* and *Acetobacterium* (Fig. 4). Members of these genera have the ability to grow on chemolithoautotrophic conditions (with hydrogen and carbon dioxide) but also chemoorganotrophically (Sleat et al., 1985; Bes et al.,

2015; Drake and Gössner, 2015; Simankova and Kotsyurbenko, 2015). As hydrogen and carbon dioxide are both present in the IPB subsurface (Amils et al., 2014), these substrates could support the growth of acetogenic bacteria and subsequently produce acetate which has been detected as well as other organic acids at various depths from rock leacheates (Amils et al., 2014). The presence of acetate in the subsurface may represent an important source of organic matter that could support growth of heterotrophic populations.

Several isolates and 16S rRNA sequences from enrichment cultures were affiliated with genera of known fermenting bacteria. Such is the case of isolates affiliated with the genus *Bacillus*, *Macellibacteroides* (Jabari et al., 2012), *Citrobacter* (Borenshtein and Schauer, 2006), *Pleomorphomonas* (Im et al., 2006; Madhaiyan et al., 2013; Xie and Yokota, 2005), *Tessaracoccus* (Finster et al., 2009; Cai et al., 2011), *Cellulomonas* (Christopherson et al., 2013; Stackebrandt and Schumann, 2014), *Microbacterium* (Evtushenko and Takeuchi, 2006; Ohta et al., 2013) or *Propionicimonas* (Akasaka et al., 2003) or 16S rRNA sequences affiliated with the genera *Sedimentibacter* and *Sporacetigenium* from T1.2MG culture, for which fermentative metabolism has also been reported (Chen et al., 2006; Lechner, 2015). Furthermore, some species of genus *Bacillus* and the majority of *Clostridium* spp. have been described that can also produce hydrogen as a fermentation product (Logan and Vos, 2015; Rainey et al., 2015). Therefore, the isolation of *Bacillus* sp. T2.9-1 in this study, as well as the recent isolation by us of a novel *Clostridium* strain (isolated under anaerobic conditions with R2A media) both from the IPB subsurface, supports the hypothesis that if fermentable substrates exist in the deep subsurface, fermentative bacteria present in the rock cores might carry out this type of metabolism and possibly contribute to generate hydrogen, which in turn could be used by other chemolithotrophic microorganisms (Sakai et al., 2007).

The isolation of strain T2.22MG-35 affiliated with the genus *Desulfovibrio*, as well as the detection of sequences affiliated with members of the genus *Desulfosporosinus* from rocks of 63.55 mbs enriched with acetate and N_2/CO_2, show the presence of sulfate reducing bacteria inhabiting the IPB deep subsurface. Members of both genera are known for their capability to grow autotrophically (on hydrogen and sulfate) as well as having a fermentative metabolism (Kuever et al., 2015; Hippe and Stackebrandt, 2015). Trace sulfate levels in the form of magnesium salt on the basal media as well sulfate leached from the rock core in the enrichment cultures may have supported the enrichment of sulfate reducing populations. Yet, we should also consider that while members of both these genera have been described as sulfate reducing bacteria, an alternative metabolic pathway may have played a role in enrichment and isolation of members of these genera (e.g. fermentation of trace organic compounds in the medium). The deep subsurface is an oligotrophic environment, as such, indigenous bacteria isolated from these ecosystems are expected to be able to grow with minor quantities of nutrients and energy sources available.

It is interesting the presence of sequences affiliated with the genus *Rhodoplanes* from 63.55 m (BH11) as well as the isolation of strains related to *Rhodoplanes* genus from BH10 at several depths (i.e. 284 m, 414 m, 492.6 m), which indicates a ubiquitous distribution in the IPB subsurface. *Rhodoplanes* species are known purple non-sulfur bacteria, characterized by having diverse metabolic capabilities, being able of both phototrophic and chemotrophic growth (Hiraishi and Imhoff, 2015). It will be interesting in future studies to explore the physiology of the *Rhodoplanes* strains isolated on this study from dark and anoxic habitats. In fact, so far the possible role in the subsurface of many of the genera isolated or detected by pyrosequencing in this study is unclear. Nevertheless, for the isolated strains, further studies on their physiological and biochemical characterization are required to further elucidate the strategies and metabolic potential that could possibly enable these strains to inhabit subsurface environments.

Recently the complete genome of strain *Tessaracoccus* sp. strain T2.5-30 isolated in this study has been sequenced (Leandro et al., 2017), as well as a draft genome of strain T2.26MG-112.2 have also been obtained. Analysis and comparison with the genomes of other members of these genera should shed light on their role in the IPB subsurface.

Isolation on roll tubes did not result in the isolation of methanogenic archaea. The resistance to isolation of methanogenic archaea could be related with the impossibility in reproducing essential key components present in the enrichment culture, e.g. present in the mineral matrix or a byproduct of the metabolism from other populations that were co-enriched. Furthermore, slow growth might also account for difficulty in isolations. Indeed, difficulty in isolating methanogens have been widely reported in many studies, with slow growth rates and fastidious growth conditions frequently indicated as causes for failure in growth (Lü and Lu, 2012). Nevertheless, pyrosequencing analysis of the archaeal 16S rRNA gene indicated the enrichment of archaea related to the genus *Methanosarcina* in all the three studied cultures, i.e. in cultures enriched using as substrate either acetate plus N_2/CO_2, a mix of propionate/butyrate/methanol plus N_2/CO_2 or an atmosphere of H_2/CO_2, as well as the enrichment of archaea affiliated with Rice Cluster I and the genus *Methanocella* (Table 4) from a culture supplemented a mixture of propionate/methanol/butyrate plus N_2/CO_2.

Members of the *Methanosarcina* genus are known to have a more versatile carbon metabolism than other methanogenic taxa, being able to use a wider range of substrates (i.e. acetate, $H_2:CO_2$ or methyl compounds) (Kendall and Boone, 2006). The detection of a positive signal by microarray analysis to members of the family *Methanosarcinaceae* has been previously reported in samples from the deep subsurface at Peña de Hierro (Puente-Sánchez et al., 2014). Archaea affiliated with the *Methanosarcina* genus has also been described in other deep subsurface environments and two novel species *M. horonobensis* and *M. subterranea* were both

isolated from a deep terrestrial environment (Shimizu et al., 2011, 2015). On the other hand, *Methanocella* is the solely genus so far characterized on the family *Methanocellaceae* in the order *Methanocellales*.

The order *Methanocellales* was previously recognized as the group Rice Cluster I (RC-I) (Sakai et al., 2007). The RC-I represented a lineage of uncultured archaea which are dominant in rice paddy soils. Members of the RC-I lineage remained uncultured for several years until in 2007 Sakai and colleagues developed a co-culture approach that allowed isolation and subsequent characterization of the first cultured member of this group, *Methanocella paludicola* (Sakai et al., 2007, 2008). The key to enrichment and isolation of *M. paludicola* SANAE[T] was the utilization of a co-culture approach with a syntrophic bacteria, which grew on oxidation of propionate and produced hydrogen as a byproduct. The bacteria used was a slow grower, therefore it provided low concentration of hydrogen over time, similar to the concentrations encountered in natural habitats, which allowed the enrichment of *M. paludicola* SANAE[T] (Sakai et al., 2007). To date, only three strains of *Methanocellales* have been obtained in pure culture, namely *M. paludicola* (Sakai et al., 2007), *M. arvoryzae* (Sakai et al., 2010) and *M. conradii* (Lü and Lu, 2012). The three isolated strains are hydrogenotrophs. Addition of acetate has been reported necessary as a carbon source for growth.

In this work, in culture T2.22MG, rock cores samples were enriched in a basal media supplemented with a mixture of propionate/methanol/butyrate plus N_2/CO_2 to promote simultaneously the culture of bacteria growing on oxidation of the small fatty acids and the enrichment of methanogens either growing on methanol or hydrogen resulting from bacterial fermentation of the supplemented fatty acids. The enrichment in populations affiliated with *Methanocella* and Rice Cluster I, possibly correlated with the co-enrichment with a hydrogen producing syntrophic bacteria. In fact, it was identified in this culture a minor enriched bacterial phylotype that has been affiliated with the genus *Syntrophomonas* (Fig. 4). Members of *Syntrophomonas* are characterized by their ability for syntrophic anaerobic oxidization of fatty acids (e.g. butyrate and other longer saturated fatty acids up to C18) (Sekiguchi, 2015). *Syntrophomonas* spp. can't oxidize fatty acids, a reaction which is thermodynamically unfavorable, unless hydrogen (a byproduct) is removed. Therefore these bacteria occur in nature on syntrophy with hydrogen-using microorganisms such as hydrogenotrophic methanogens (Müller et al., 2010). To best of our knowledge this is the first report of the presence of members of the *Methanocellales* on rock cores from subsurface environments. Much is still necessary to be learned about this intriguing group and the identification of members enriched from rock samples retrieved from 453.3 m deep into the subsurface of the Iberian Pyritic Belt provides further information about the ecological distribution of these archaea.

In this study the enrichment in methanogenic activities, the massive assay effort on isolation of microorganisms from hard rock of both strict and facultative anaerobic

isolates as well as the identification by high throughput sequencing of diverse populations of facultative and strict anaerobic bacteria and methanogenic archaea, provided an important piece of information towards the global understanding of microbial diversity in the deep subsurface of the Río Tinto aquifer at the Iberian Pyritic Belt. The occurrence in rocks of microbial strains affiliated with microorganisms with metabolic potential for fermentation (e.g. *Bacillus* sp. or *Cellulomonas* sp.), isolates and/or sequences affiliated with known methanogenic archaea (e.g. *Methanosarcina* sp., *Methanocella* sp.), acetogenic bacteria (e.g. *Acetoanaerobium* sp., *Sporomusa* sp., *Acetobacterium* sp.) or sulfate reducing bacteria (e.g. *Desulfovibrio* sp., *Desulfosporosinus* sp.), suggests a potential important role for these microorganisms on the cycling of elements such as carbon and sulfur through the subsurface ecosystem. Moreover, this data also indicates that diverse microbial populations occur within the subsurface of Río Tinto aquifer.

Declarations

Author contribution statement

Tania Leandro: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nuria Rodriguez: Performed the experiments.

Patricia Rojas: Analyzed and interpreted the data.

Jose L. Sanz, Ricardo Amilsa: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Milton S. da Costa: Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

The 16S rRNA bacterial gene sequences obtained in this study have been deposited at the DDBJ/ENA/GenBank under the accession numbers [MF361863−MF361869,

MF361871, MF361873−MF361879; MF361881, MF361882, MF361883, MF361885, MF361886, MF361888, MF361889, MF361890, MF361893, MF361894, MF361895]. The raw sequences from pyrosequencing obtained in this study were deposited in the National Center for Biotechnology Information (NCBI) under accession numbers SRR6186122 − SRR6186127.

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