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Distinct deep subsurface microbial communities in two sandstone units separated by a mudstone layer

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ABSTRACT: Deep subsurface microbial communities are more abundant in coarse-grained sedimentary rocks such as sandstones than in fine-grained mudstones. The low porosity and low permeability of mudstones are believed to restrict microbial life. Then, it is expected that distinct, isolated microbial communities may form in sandstones separated by mudstones. In this context, the connectivity between microbial communities in different sandstone units can be investigated to infer evolutionary patterns of diversification in space-time, which may potentially contribute with relevant data for analyses of hydraulic connectivity and stratigraphic correlation. In this work, we used high throughput DNA sequencing of a ribosomal 16S gene fragment to characterize the prokaryotic communities found in Permian sandstone samples of the same core that are separated by one mudstone interval, in the Charqueadas coal field, Parana Basin (Southern Brazil). Our samples were collected at ~300 m deep, in porous sandstones separated by a thick mudstone package. Differences in the bacterial community structure between samples were observed for the classified OTUs, from phylum to genus. Molecular biology might be further applied as a possible tool to help to understand the spatial and temporal distribution of depositional facies, and the efficiency of low permeability rocks to compartmentalize reservoirs. Ongoing studies aim to extend the present investigation into further analyses regarding lateral changes in microbial communities present in the same sandstone units.

Key words: mudstone, Parana Basin, high throughput sequencing, hydraulic connectivity

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1. INTRODUCTION

It has been demonstrated that deep subsurface microbial communities are more abundant in coarse-grained sedimentary rocks such as sandstones than in fine-grained mudstones (Fredrickson et al., 1997). The low porosities and extremely low permeability of the latter are believed to restrict microbial life (McMahon and Chapelle, 1991; Sharma and McInerney, 1994; Wilkins et al., 2014). Mudstones are, however, documented sources of nutrients (diffusion of organic matter or its fermentation products) for adjacent sandstones (Krumholz et al., 1997). The restrictions for microbial life found in fine-grained rocks imply that: (i) distinct, isolated microbial communities may form in sandstones separated by mudstones, and (ii) microbial communities may change in sandstones when close to mudstones (Krumholz et al., 1997). The similarities and differences in microbial communities could be used therefore to understand the spatial and temporal distribution of depositional facies, and the efficiency of fine-grained, low permeability rocks to compartmentalize reservoirs. The connectivity between microbial communities in different geological units can be investigated through phylogeographic approaches using molecular markers to infer evolutionary patterns of diversification in space-time (Riddle et al., 2008).

Molecular biology techniques have the robustness to become

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therefore a significant tool to be applied as a complement to conventional stratigraphic correlation, reservoir compartment analysis, and dating methods of sedimentary rocks. In this context, we characterized prokaryotic communities found in two Permian sandstone samples of the same core that are separated by one mudstone interval, in the Charqueadas coal field, Parana Basin (Southern Brazil).

2. MATERIAL AND METHODS

2.1. Samples and Study Area

Three samples were collected from a drill core (CEPAC-01) intended for coal bed methane exploration in a region with no significant regional faults and fractures (Santarosa et al., 2013) in the Charqueadas coal field, Paraná Basin, Southern Brazil (Fig. 1). The samples obtained for this study belong to the Rio Bonito and Palermo formations (Guatá Group, Lower Permian) and consist of coastal and shallow marine deposits, respectively (Holz et al., 1999). The Rio Bonito Formation, in particular, contains Brazil's most important coal deposits and is formed by sandstones, mudstones, and paraconglomerates associated with the coal seams. The coal-bearing strata include both coal seams and coaly shale intercalations deposited in subaquatic or swamp conditions (Correa da Silva, 2004). The Palermo Formation occurs on top of the Rio Bonito Formation and is formed by quartzose, fine to conglomeratic sandstones interfingered with bioturbated siltstones, which shows both lenticular and flaser structures.

Two massive sandstone samples with similar grain size (fine to medium) from the Rio Bonito and Palermo Formations with preserved formation water were collected at 278 m (CEPAC_A) and 319 m (CEPAC_C) below the surface, above and below a 32 m thick mudstone interval, respectively, during drilling of the well CEPAC-01 (Fig. 2). A representative sample of the mudstone

Fig. 1. Location map of study area showing a coal bed methane exploration well in the Charqueadas coalfield, Paraná Basin, southern Brazil. Samples were obtained from the CEPAC-01 well (red circle).

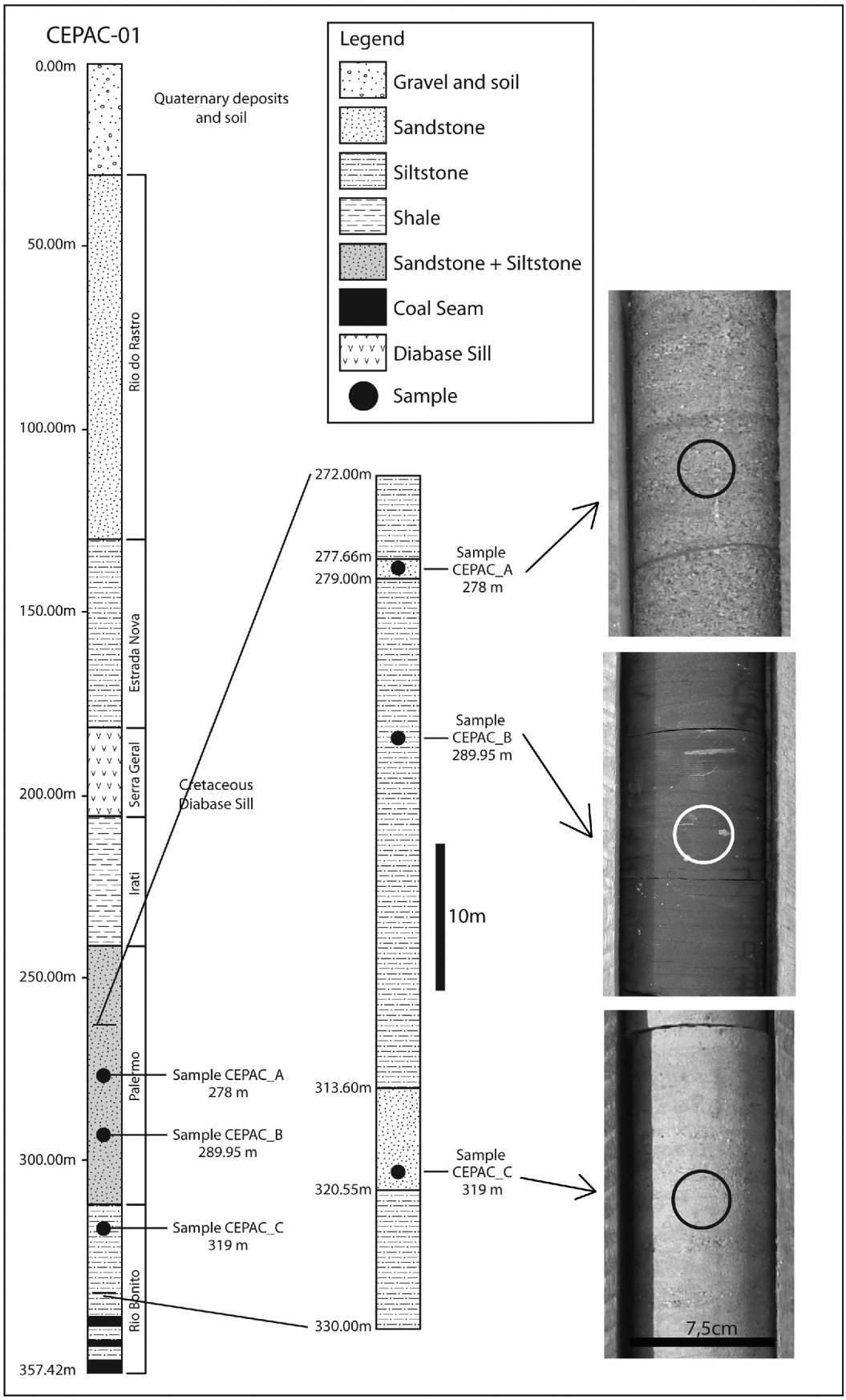

Fig. 2. Lithological log for CEPAC-01 well showing the positioning of the three samples obtained for this work. See Figure 1 for location of the well.

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interval was also collected at 289.9 m deep (CEPAC_B). The well was logged by continuous, downhole resistivity and natural gamma-ray measurements (Fig. 2). Two air-permeability measures were executed in the CEPAC-01 well in the context of coal bed methane exploration (sandstone at 317 m and mudstone at 337 m). Steady-state permeability was determined at 800 psi confining pressure using the Frank Jones steady-state Permeameter at the Wheatherford Laboratories Brazil. As drilling and sampling from subsurface may involve different methods, and drilling fluid may be circulated inside the well, samples were collected from the inside of the core, avoiding external contaminations. Samples were kept on dry ice during sampling and later stored at –20 °C for further DNA isolation.

2.2. DNA Isolation, 16S rRNA Gene PCR and High Throughput Sequencing

For DNA isolation, rock powder was obtained using a sterile friction blade to remove fragments from a previously fractionated rock with a mallet and drill for each particular type of rock material. DNA extractions were performed from 400 mg of adequate granulometry rock powder using QIAmp kit Stool Mini Kit (QIAGEN). DNA extractions were performed in a UV-sterilized PCR workstation, located in a laboratory area isolated from PCR products, microbial clones and genomic DNA from potential contaminants. Dedicated instruments (e.g., micropipettes) and consumables were also employed to minimize the chance of contamination with exogenous DNA sources.

Extracted DNAs obtained from samples were subjected to PCR amplification from the 16S rRNA gene, using the primers 515F and 806R as described by Bates et al. (2011). PCR amplicons were purified using Agencount AMPure Beads (Beckman Coulter), following the manufacturer's protocol. Sequencing libraries construction was performed as described in the Ion Plus Fragment Library for short amplicons (≤ 350 pb) from an initial amount of 100 ng of PCR product. Sequencing was conducted on an Ion PGM System (Thermo Fisher) using the Ion 316 chip, following manufacturer's instructions.

2.3. High Throughput Sequencing Analysis

The 16S rRNA reads generated using high throughput sequencing were submitted to quality control that retained sequences with a minimum length of 100 bp and trimmed to remove low-quality bases for a minimum Phred score of 30 using PRINSEQ (Schmieder and Edwards, 2011). The remaining sequences were dereplicated and sorted by decreasing read abundance and then filtered to exclude singletons using USEARCH v7.0.1090 (Edgar, 2010). Clusters were assembled using a minimum identity of 99%, and chimeras were removed using RDP reference database (Cole et al., 2014). The taxonomic assignment was obtained using QIIME v1.8.0 (Caporaso et al., 2010). OTUs were selected based on 97% sequence similarity and taxonomic data were achieved through the classification algorithm using the GreenGenes 13.8 (DeSantis et al., 2006). Alpha diversity metrics were calculated using QIIME v1.8.0. Multiple rarefactions were performed for Chao1 (species richness), Shannon (diversity), and Simpson_e (evenness). Sequences have been deposited under the accession number PRJNA377619 in the NCBI BioProject database.

3. RESULTS

To characterize the prokaryotic diversity present in sandstones separated by mudstone, CEPAC_A and CEPAC_C (sandstones) and sample CEPAC_B (mudstone) were analyzed. The resistivity log of the well suggest that there are no major differences in pore water salinity in both analyzed sandstones (ca. 30 Ohm-m; Fig. 2). Permeability measurements were 923 mD for the sandstone sample and 1.74 mD for the mudstone sample. Segments of the 16S rRNA gene were amplified and sequenced using high throughput DNA sequencing technology for samples CEPAC_A and CEPAC_C. It was not possible to retrieve DNA from the CEPAC_B mudstone sample after five trials because the amount of DNA extracted was lower than the detection limits. After trimming low-quality bases and removing short reads, a total of 360,510 16S rRNA sequences were employed in the downstream analyses: 139,972 sequences from CEPAC_A and 220,538 sequences from CEPAC_C (Table 1).

The classification of 16S rRNA sequences indicated that the prokaryotic communities present in the samples might be distinct from each other. Although CEPAC_C presented a much higher number of observed OTUs compared to CEPAC_A, the Shannon (diversity) and Simpson_e (evenness) indexes, as well as the nonparametric richness estimation from Chao1 were highest for CEPAC_A sample (Table 1).

The bacterial domain was distributed within 6 phyla, 17 classes, 29 orders, 61 families, and 85 genera OTUs. No archaeal sequences were retrieved. All the six bacterial phyla were identified

Table 1. Overview of the number of sequences in samples from different depths and alpha diversity data from observed operational taxonomic units (OTUs), Shannon (diversity), Simpson_e (evenness), and Chao1 for nonparametric richness estimation

Sample	Number of sequences	Observed OTUs	Shannon	Simpson_e	Chao1
CEPAC_A	139,972	185	5.343	0.132	207.4
CEPAC_C	220,538	171	4.825	0.094	200

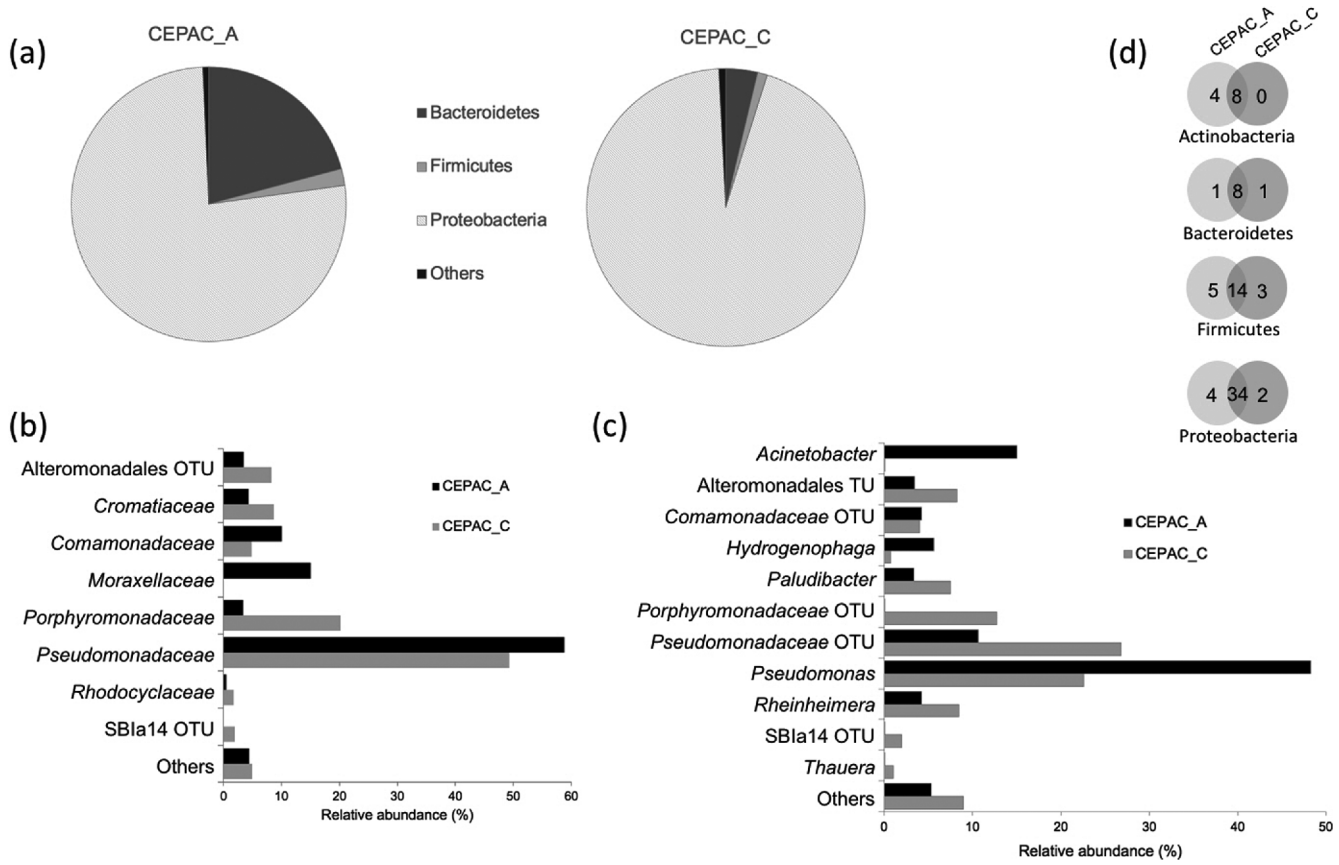

Fig. 3. Relative abundance of dominant bacterial groups (≥ 1% of the total sequences) in sandstone samples separated by a mudstone: (a) at phylum level; (b) at family level; and (c) genus level. (d) Venn diagrams showing the unique and shared bacterial OTUs between CEPAC_A and CEPAC_C samples.

in both samples, and Proteobacteria was the dominant phylum, followed by Bacteroidetes and Firmicutes. At the phylum level, rare taxa (less than 1% of the total reads) are represented by Actinobacteria, Cyanobacteria and Verrucomicrobia ("others" in Fig. 3a). At the family level, only eight OTUs were present in more than 1% in at least one sample. The family *Pseudomonadaceae* (class Gammaproteobacteria) was the most abundant in both CEPAC_A and CEPAC_C samples. In CEPAC_A, *Pseudomonadaceae* and *Moraxellaceae* (Gammaproteobacteria) were responsible for more than 73% of the total reads, with 59% and 14% of frequency, respectively (Fig. 3b). Differently, in CEPAC_C *Moraxellaceae* was not detected. *Pseudomonadaceae* presented 49% of the total reads in this sample and was followed by *Porphyromonadaceae* (Class Bacteroidia), which was more abundant in this sample in comparison to CEPAC_A, with 20.2% and 3.4% of the total reads, respectively (Fig. 3b). At the genus level, eleven from the 85 OTUs were more abundant than 1% of the total reads. Genera *Pseudomonas* and *Acinetobacter* (phylum Proteobacteria, class Gammaproteobacteria) dominated the sample CEPAC_A, with 48.1% and 15% of the total reads, respectively (Fig. 3c), whereas *Acinetobacter* was observed 181 times more in CEPAC_A than in the CEPAC_C. Moreover, The Betaproteobacteria genus *Hydrogenophaga* (family *Comamonadaceae*) was seven times more abundant in CEPAC_A than in CEPAC_C.

In sample CEPAC_C, the most abundant microorganism was an OTU belonging to the family *Pseudomonadaceae* (26.7%), followed by *Pseudomonas* (22.6%). OTUs belonging to the family *Porphyromonadaceae* was found 236 times more abundant in CEPAC_C than in CEPAC_A. The betaproteobacterial OTU SBIa14 and the genus *Thauera* were found 31 and 52 times more abundant in CEPAC_C than in CEPAC_A, respectively (Fig. 3c).

The genera *Desulfomicrobium* and *Proteus* were found exclusively at CEPAC_C, and the genera *Mycoplana*, *Dokdonella* and the OTUs from the families *Bacteriovoracaceae* and *Campylobacteriaceae* were found solely at the CEPAC_A sample. Moreover, the Actinobacteria genera *Micrococcus*, *Nocardiopsis*, *Xylanimicrobium* and an OTU from the family *Microbacteriaceae* were also present only at CEPAC_A. The Bacteroidetes genera *Hymenobacter* and *Pedobacter* were found exclusively at the CEPAC_C and CEPAC_A samples, respectively. The Firmicutes genus *Paenibacillus*, the OTUs from the family *Bacillaceae* and the Class AHT28 were observed solely in CEPAC_C. The Firmicutes genera *Vagococcus*,

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Clostridium, *Acetobacterium*, *Pelosinus* and an OTU from the family *Carnobacteriaceae* were present only at the CEPAC_A sample.

The shared bacterial OTUs for different sandstone samples can be seen in a Venn diagram (Fig. 3d). A total of 65 classified OTUs (approximately 76%) could be detected in both CEPAC_A and CEPAC_C samples. Four Actinobacteria, one Bacteroidetes, five Firmicutes, and four Proteobacteria OTUs were found exclusively in CEPAC_A. One Bacteroidetes, three Firmicutes and two Proteobacteria OTUs were retrieved solely from the CEPAC_C sample. None Actinobacteria OTU was exclusively retrieved from this sample.

4. DISCUSSION

Our work has demonstrated that there are distinct deep subsurface microbial communities living in permeable sandstones with similar pore water salinities separated by a low-permeability mudstone. This observation has also been reported for other settings, such as in the Cretaceous Mancos shale and Dakota sandstone in the USA (Krumholz et al., 1997). Although we easily obtained DNA from sandstones, the unsuccessful retrieval from the interbedded mudstone sample is possibly attributed to the constraints to microbial life occurring in fine-grained rocks owing to, for instance, restriction of microbe penetration, and inhibition of reproduction and metabolic activities (McMahon and Chapelle, 1991; Sharma and McInerney, 1994; Wilkins et al., 2014). Moreover, previous studies on continental subsurface microbiology have already observed that the number and diversity of microorganisms decrease with depth (Escudero et al., 2018a), which may also have contributed to the lack of retrieved DNA from CEPAC_B, in addition to the fine-grained feature of this sample. Regarding the samples from which we retrieved DNA and analyzed the microbial communities (CEPAC A and CEPAC C), the indexes for richness estimation (Chao1), diversity (Shannon) and evenness (Simpson_e) indicated lower values for the most in-depth sample, CEPAC C, compared to the CEPAC_A, corroborating this general observation (Escudero et al., 2018a).

Escudero et al. (2018a) surveyed several microbiological studies from deep continental subsurface and observed that in general, very few projects had performed devoted geomicrobiological drills to collect pristine samples. Indeed, many researchers have taken advantage of subterranean "artificial windows" for deep sampling in terrestrial environments. The authors also argue that the study of the subsurface biosphere through these artificial systems may have been significantly modified, harboring microbial populations that may not be representative of the native communities. In our study, we used this strategy and analyzed samples from a coal field drilling core. Nevertheless, it is essential to note that our samples were collected from the inside of the core, which may have protected them from external contaminants. Moreover, for DNA isolation, the rock powder was obtained using sterile tools for each particular type of rock material, in a UV-sterilized PCR workstation, which also may have prevented external interferences.

The studied Permian sandstone samples might be considered, from a microbiological point of view, an unknown habitat, which is still poorly explored. Even then, data in literature have shown that microbial diversity can be successfully retrieved from deep rock samples, yet it is considered a low-nutrient availability environment (Escudero et al., 2018b). In these environments, the abundance of Bacteria is in general superior to Archaea, and within Bacteria, the most commonly detected phyla correspond to Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes (Escudero et al., 2018a), which was the ones detected in our study. Also, a 16S rRNA pyrosequencing survey realized in coalmines in Australia (around 150 m depth) reported 22 microbial prokaryotic phyla, three of them from the archaeal domain, representing more than 35% of the total 16S rRNA reads, as well as few unclassified microorganisms in coalmines samples (Raudsepp et al., 2016). Guo et al. (2012) also performed a pyrosequencing analysis from samples drilled down to about 700 m (also Permian coal-bearing samples) in China, finding fourteen bacterial phyla and the archaeal phylum Euryarchaeota. This survey has also revealed the genera *Hydrogenophaga* and *Acinetobacter* as dominant in coal samples (Guo et al., 2012).

The most abundant OTUs found in CEPAC_A belong to the proteobacterial genera *Pseudomonas* and *Acinetobacter*. Both genera belong to the same bacterial order (Pseudomonadales) and are reported as occurring in a broad range of samples and environments. Their versatile metabolic characteristics allow these genera to catabolize a wide range of natural compounds (Silby et al., 2010; Jung and Park, 2015). Moreover, the genus *Pseudomonas* is described as able to degrade coal-associated kerogen and associated solvent-extractable material (An et al., 2013; Raudsepp et al., 2016). *Acinetobacter*, a widespread soil genus related with biodegradation of polycyclic aromatic hydrocarbons (PAHs), was found in contaminated groundwater from a coal-mining area in China (Shao et al., 2015), as well as in disturbed coal mine environments (Raudsepp et al., 2016). Interestingly, the genus *Pseudomonas* was found to produce a rhamnolipid biosurfactant in the formation water of a coalbed (Singh and Tripathi, 2013) and *Acinetobacter* as a producer of the biosurfactant RAG-1, a known industrial surfactant that enhances hydrocarbons mobility (Siegert et al., 2013).

Differences in the community structure between samples were observed for the classified OTUs, from phylum to genus

level. In porous and permeable (i.e., 923 mD) sandstones our samples were separated by a thick, low permeability (i.e., 1.74 mD) mudstone package. These rocks probably contributed to reducing the vertical flow of microorganisms between above and below, and/or partially isolated specific microbial populations, which can be observed using a high throughput DNA sequencing approach. Although the depth difference of 41 m might be large enough to differentiate the microbial community structures especially for the vertical direction, still the molecular biology might be further applied as a possible tool to help the identification of reservoir compartments and suggest hydraulic connectivity or isolation among these units. Although the microbial communities change with increasing burial depth and it is related to several environmental factors, such as conditions of nutrient supply, fluid flow existence, and temperature pressure changes (Escudero et al., 2018b), ongoing studies aim to extend the present investigation into further analyses regarding lateral changes in microbial communities present in the same sandstone units. Progress of this type of research will significantly contribute to this new field of investigation, in which the hydraulic connectivity between strata is supported by microbiological and molecular biology data. Besides, this study has far-reaching implications, as we demonstrated that fine-grained rocks are indeed barriers for microorganisms in the deep subsurface and, therefore, might be relevant, for instance, for the dispersion and control of contaminants, and bioremediation strategies in the subsurface.

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