Bacterial diversity in bentonites, engineered barrier for deep geological disposal of radioactive wastes

Margarita Lopez-Fernandez[1], Andrea Cherkouk[2], Ramiro Vílchez-Vargas[3], Ruy Jauregui[4], Dietmar Pieper[4], Nico Boon[3], Iván Sánchez-Castro[1], Mohamed L. Merroun[1]

[1]Department of Microbiology, University of Granada, Granada, Spain

[2] Helmholtz-Zentrum Dresden-Rossendorf, Institute of Resource Ecology, Bautzner Landstrasse 400, 01328 Dresden, Germany

[3]Laboratory of Microbial Ecology and Technology, Ghent University, Ghent, Belgium

[4]Helmholtz Centre for Infection Research, Braunschweig, Germany

Corresponding author: Margarita Lopez-Fernandez

Email: margaritalopez@ugr.es

Phone: +34 958249331, Fax: +34 958249486

Abstract

The long-term disposal of radioactive wastes in a deep geological repository is the accepted international solution for the treatment and management of these special residues. The microbial community of the selected host rocks and engineered barriers for the deep geological repository may affect the performance and the safety of the radioactive waste disposal. In this work the bacterial population of bentonite formations of Almeria (Spain), selected as reference material for bentonite engineered barriers in the disposal of radioactive wastes, was studied. 16S rRNA gene-based approaches were used to study the bacterial community of the bentonite samples by traditional clone libraries and Illumina-sequencing. By both techniques the bacterial diversity analysis revealed similar results, with phylotypes belonging to 14 different bacterial phyla: *Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Bacteroidetes*, *Choloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus, Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria, Nitrospirae*, *Verrucomicrobia* and unknown. The dominant groups of the community were represented by *Proteobacteria* and *Bacteroidetes*. A high diversity was found in three of the studied samples. However, two samples were less diverse and dominated by β–proteobacteria.

Keywords

Spanish bentonite, bacterial diversity, Illumina sequencing, Cloning and sequencing

Introduction

Many countries are considering long-term disposal of nuclear waste in a deep geological formation, encapsulated in metal container, surrounded by a bentonite-engineered barrier, and emplaced in the host rock [1-3]. In Spain, bentonite formations located in Almeria have been intensely studied due to their possible use as natural analogue of the bentonite-engineered barrier in the deep geological repository for radioactive waste. In addition, these bentonite formations were selected as Spanish reference material because they are well characterized from mineralogical, geochemical and technological points of view [4]. Clay is not only a candidate as backfill or sealing material but also a suitable host rock for a high-level radioactive waste repository in other European countries as for example Opalinus clay in Switzerland, Boom clay in Belgium, and Bure clay in France. In some of these clay formations, microbiological studies were performed to get information about what kind of microorganisms are present, about their viability and activity. Occurrence of viable indigenous microbes, including sulphate-reducing bacteria and also some isolated strains belonging to genus *Sphingomonas*, was evidenced in Opalinus Clay at the Mont Terri Underground Research Laboratory by culture-based methods [5-6]. A multidisciplinary approach was performed to study the microbial diversity in Boom clay formation, a deep-subsurface clay deposit in Mol, Belgium [7]. In the Meuse/Haute-Marne Underground Research Laboratory located at Bure (300 km east of Paris) the Callovo-Oxfordian argillite formation was evaluated for its use as a potential host rock for a high-level radioactive waste repository in France [8] and its microbial diversity was studied twice by Urios *et al*. [9]. The bacterial diversity found at the French formations was dominated by *Firmicutes*, *Actinobacteria* and *Proteobacteria* [9]. It is of great importance to know what microbes occur in the selected Spanish clay formation because present microbes could impact the rate of processes implicated in 1) metal corrosion, 2) transformation of clay minerals, and 3) radionuclide migration and transport [10]. All these processes may impact the safety of the repository by compromising its isolation and containment functions [10]. Microorganisms can potentially affect radionuclide migration by various processes including biosorption, biomineralization, intracellular accumulation, biotransformations, etc. [11-17]. In addition, microbial occurrence can influence the release of radionuclides by changing geochemical conditions (especially pH and Eh), by producing organic complexes [18]. Moreover, microorganisms can also affect the conditions in the repository by microbial reduction or dissolution of the clay minerals [19], by microbial production or consumption of gases, which can generate an overpressure and form fractures [20] as well as by microbial

degradation of organic compounds, which can be parts of the radioactive waste or container material, affecting the longevity of the metal waste container in the repository [21]. However, in the case of Spanish clay formations, so far only one investigation of the presence of microorganisms was performed. Culture-dependent analysis of microbial diversity from these clay formations were presented in Lopez-Fernandez *et al*. [22]. Bacteria from different phyla were isolated, whereas representatives from *Proteobacteria*, *Firmicutes* and *Actinobacteria* were dominant. In addition, a pigmented yeast strain namely *Rhodotorula mucilaginosa* BII-R8 was also recovered [22]. Nevertheless, this approach is limited as a small percentage of natural microbial populations can be isolated and studied in the laboratory due to the limited knowledge about their nutrient requirements and other life-necessities [23-24]. Different methods based on 16S ribosomal gene sequences have been used to characterize the microbial diversity since beginning of the 80´s and they have revealed a tremendous prokaryotic diversity which was overlooked by traditional culture enrichment techniques [23]. Next Generation Sequencing (NGS) is a good method to study the richness and evenness of a prokaryotic community. Illumina-sequencing platform allows a full characterization of the bacterial community, with the major advantage of obtaining thousands of gene sequences [25]. However, this new technology is limited by the short length of the reads. Therefore, to improve the taxonomical affiliation of the system studied, analysis of 16S rRNA gene clone libraries might be applied. The latter method provides longer reads, analyzing almost the full 16S rRNA gene, which makes allowance to go deeper into the taxonomy, up to genera and species. However, it is necessary to analyze a high amount of clones per sample to reach sufficient reads to completely characterize the most abundant representatives of the community [26]. The work presented in this study is focused on the bacterial diversity analysis of the already mentioned Spanish bentonites, by two different culture- independent molecular approaches based on the 16S ribosomal RNA gene analysis via (i) Illumina sequencing platform and (ii) cloning and sequencing, to get a better knowledge about what kind of microorganisms are present and how these microorganisms can potentially influence the performance of the nuclear waste repository.

Materials and methods

Description of bentonite samples

Five bentonite samples were collected from clay formations from three different sites in Almeria, in the south-east of Spain during March 2011 (Fig. 1). Bentonites from these clay deposits are best described as

a natural analogue of the bentonite-engineered barrier in the context of deep geological disposal of radioactive waste for their good compaction properties [4]. Two samples called BI-2 and BI-3 were collected from El Cortijo de Archidona, site BI. Bentonites from this area are mainly made up of ash and pumice fragments, with a predominance of green and blue colours (Fig. 1b). They are very plastic materials that show extrusion signals from the reactivation of the fault after the bentonite formation process [27]. Another sample called BII-2 was collected from the acid area of El Toril, site BII, which is 50 meters to the north of El Cerro del Toril. This area results from an acid alteration of the original deposits caused by physical, chemical and mineralogical changes in the bentonite material [28]. The superficial area of the fault contains jarosite, an iron sulfate mineral responsible for its ocher coloration, as it is shown in Figure 1c. The last two samples called BIV-2 and BIV-3 were collected from Los Trancos, site BIV, over the Carboneras fault, at the south-east of Carboneras. This is the area with the highest presence of bentonites in this region of Almeria (Fig. 1a). Samples called BI-2, BII-2 and BIV-2 were taken from the surface; whereas samples BI-3 and BIV-3 were taken from a depth of twenty centimeters. All samples were collected under sterile conditions and stored frozen at −80 ºC until used for further analysis.

Geochemical and Mineralogical Analyses

X-ray diffraction (XRD) characterization of the three bentonite sites was done as described in Lopez-Fernandez *et al*. [22]. Composition of the major elements of each sample was determined by X-ray fluorescence spectroscopy (XRF), on pressed pellets made of 1:10 lithium tetraborate dilution. A portion of this pellet was burned at 1000 ºC for 1 hour to calculate the Loss On Ignition (LOI). The instrument used for the XRF measurements was Philips-Magix Pro (PW-2440), Netherland. De Jongh model [29] was used to convert the X-Ray intensities into concentrations (Philips software). Trace elements content of the samples was determined in triplicate by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) using NexION 300d spectrometer (Perkin Elmer) with a precision of ± 5% for an analyte concentration of 10 ppm. Thus, 0.1 g of sample powder was digested with a mixture of HNO_3 and HF in a Teflon-lined vessel, evaporated to dryness, and subsequently dissolved in 100 ml of 4 vol. % HNO_3.

Organic carbon content and pH measurements

Total organic carbon of the bentonite samples was determined by wet oxidation according to the optimized Walkley and Black's method [30], a standard method to measure organic carbon in agricultural

soils described by Mingorance *et al*. [31]. The pH was measured on a 1:5 (w/v) soil:water suspension. One part of clay was combined with five parts of distillated water. Suspensions were shaken for 10 minutes and after decantation, the pH of the supernatant was measured three times using a Crison pH-meter (MicropH 2002). The instrument was previously standardized against pH 4.00, 7.02 and 9.18 commercial reference solutions. The reported accuracy was of ± 0.02 pH units.

DNA Extraction and Ribosomal Intergenic Spacer Analysis (RISA)

Total DNA was recovered from 10 g of bentonite soil sample by a method developed by Selenska-Pobell and co-workers [32]. This method uses SDS to lyse the cells and release bacterial DNA instead of a mechanical lysis step often used in commercial DNA extraction kits [33]. The very effective direct lysis of microorganisms in environmental samples is combined with the precipitation of the extracted DNA with polyethyleneglycol, and the final purification steps based on the use of AXG-100 cartridges (Macherey-Nagel, Düren, Germany). The resulting DNA pellet was dissolved in 35 µl of sterile Milli-Q water. For screening the bacterial communities, Ribosomal Intergenic Spacer Analysis (RISA) with the primers $16S_{969f}$ (5'-ACG CGA AGA ACC TTA C-3') and $23S_{130r}$ (5'-GGG TTN CCC CAT TCG G-3') [34] was performed as described by [32].

Illumina sequencing

Total DNA extracted was also sequenced by Illumina following the procedure of Camarinha-Silva *et al*. [35]. Hypervariable region V5-V6 of the 16S rRNA gene was amplified by using universal primers based on $16S_{807f}$ and $16S_{1050r}$ [36]. Resulting PCR products were amplified using sequencing primers for V5-V6 region, the forward primer contains a 6-nt barcode [37] and a 2-nt CA linker [38]. Forward and reverse primers comprised sequences complementary to the Illumina specific adaptors to the 5′-ends. Amplification was performed in a total volume of 50 µl with 5x PrimeSTAR TM buffer (Clontech Laboratories, Mountain View, CA, USA), containing each deoxynucleoside triphosphate at a concentration of 2.5 mM, each primer at a concentration of 0.2 µM, 1 µl of template DNA and 0.5 µl PrimeSTAR TM HS DNA polymerase (2.5 units, Clontech Laboratories, Mountain View, CA, USA). An initial denaturation step of 95 °C for 3 min was followed by 15 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 45 s. One microlitre of this reaction mixture served as template in a second PCR performed under the same conditions as described above, but for 10 cycles using PCR primers designed to integrate the sequence of the specific Illumina multiplexing sequencing

primers and index primers. Non-template controls (using water as template) were performed and were free of any amplification products after both rounds of PCR. PCR amplicons were verified by agarose gel electrophoresis, purified using Macherey-Nagel 96-well plate purification kits (Macherey- Nagel, Düren, Germany) following the manufacturer's instructions and quantified with the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen, Darmstadt, Germany). Libraries were prepared by pooling equimolar ratios of amplicons (200 ng of each sample), all having been tagged with a unique barcode. In total, five libraries were prepared. To remove any contaminants or PCR artefacts, each library was precipitated on ice for 30 min after addition of 20 µl of NaCl (3M) and three volumes of ice-cold 100% ethanol. The precipitated DNA was centrifuged at 16000 rpm for 30 min at 4 °C. The supernatant was removed, the pellet air dried, resuspended in 30 µl of double distilled water and separated on a 2% agarose gel. PCR products of the correct size were extracted and recovered using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Libraries were sent for paired-end sequencing on a MiSeq System Sequencer (Illumina, California, USA), obtaining 92832 sequences of 280 nt length. R-program (with vegan and phyloseq packages) was used to normalize to the minimum the pool of sequences, for plotting the rarefaction curves and for calculating the diversity indexes. The sequences were annotated using SILVA Incremental Aligner (SINA) [39].

Clone library analysis

16S rRNA gene fragments were amplified by PCR in a reaction mixture of 20 µl, containing 1 µl of (≈100ng/µl) DNA template, 2.5 mM of $MgCl_2$ Solution, 125 µM of each of the four deoxynucleoside triphosphates, 350 nM each of the forward and reverse primers and 1 unit Go Taq® DNA Polymerase with the corresponding Go Taq® Flexi Buffer (Promega, Mannheim, Germany)**.** The primers used for this reaction were the bacterial universal primers $16S_{8F}$ (5'-AGAGTTTGATCCTGGCTCAG-3') [40] and $16S_{1492R}$ (5'-TACGGYTACCTTGTTACGACTT-3') [41]. The PCR amplifications were performed as described by [42], using 2 µl of reaction mixture obtained combining three parallel replicates. A total of 100 single white colonies were randomly selected. The inserted 16S rRNA gene fragments were amplified and further analyzed according to [42]. PCR products of selected clones were purified using Exo-SAP purification protocol, which uses two hydrolytic enzymes, Exonuclease I (New Englands Biolabs, U.K.) and Thermosensitive Alkaline Phosphatase (TSAP) (Promega, Germany), in a specially formulated buffer for the removal of unwanted primers and dNTPs. After adding the enzymes to the PCR product a 30 min incubation at 37 °C is following and then an enzyme inactivation at 85 °C for further 15 min. Purified

PCR products were sequenced as described by [42]. The rest of the clones, not grouped in any predominant type, were classified as individual representatives of the community.PCR products of these clones were sent to GATC Biotech (Germany) to be purified and sequenced. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 [43]. The $16S_{8F}$ and $16S_{1492r}$ sequences of each clone were aligned using software MEGA 5 (ClustalW) and compared to those from GenBank using the Basic Local Alignment Tool (BLAST) server at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were generated also with MEGA5 using the neighbour-joining algorithm and bootstrapped (500 trial replicates). The possibility of chimera formation by 16S rRNA gene sequences was checked by submitting sequences and their closest phylogenetic relative to the pintail program, version 1.1 (http://www.mybiosoftware.com). Possible chimeras were excluded from the phylogenetic analyses. 16S rRNA gene sequences of the bacterial clones were submitted to the European Nucleotide Archive, under accession numbers HG970666- HG970729 and LK023520- LK023709.

Statistical multivariate analysis (PCA)

Statistical calculations for the principal component analysis were performed in R [44] using vegan package. Geochemical, mineralogical and bacterial diversity (up to phylum level) results were used for the calculations.

Results

Sampling and characterization of the bentonites

The mineralogy of the bentonite samples is dominated by smectites, combined with minor quantities of feldspars, quartz, etc. [27]. The bentonites show different colours: white, green, red, blue, brown, and so forth, depending on the trace elements contents of the first transition series [45]. In this work we focused on three different sampling sites which were characterized from a mechanical, mineralogical and chemical point of view, as well as by the processes involved in their genesis [46]. In each sampled site, the bentonites exhibit different characteristics, due to the type of rock undergoing alteration, or chemical composition for example [47]. The XRD semi-quantitative estimation of the mineralogical composition of the studied sites revealed that smectites (montmorillonites) are the dominant mineral phase, 84%, 71% and 96% in sites BI, BII [22] and BIV, respectively (Table 1). Moreover, feldspars were detected: K-feldspar (sanidine) in site BII and plagioclase (albite) in sites BI and BIV. The presence of jarosite, an iron

sulphate mineral phase, was only detected in site BII. XRD diffractograms are shown in Online Resource 1. Elemental analysis of the bentonite samples revealed that an increased concentration of iron oxides (Fe_2O_3) and sodium oxide (Na_2O) is present in samples BI-2 and BI-3, respectively (Table 2). However, sample BII-2 showed the biggest differences compared to the rest of the samples. For example, this sample had high concentrations of potassium oxides (K_2O), iron oxides (Fe_2O_3) and phosphorus pentoxides (P_2O_5) compared with previous data published for this region [48-49]. The high content of Fe_2O_3 confirms the characteristic composition because of the presence of jarosite in sample BII-2 (Table 1). In addition, this sample had the lowest bacterial diversity as it was revealed by the rarefaction curves (Fig. 2). Comparing samples BIV-2 and BIV-3 with the rest of the samples, a ten times higher concentration of manganese oxide (MnO) was detected, although it was similar to MnO average published [50]. In addition, concentrations of minor elements were determined by ICP-MS (Online Resource 2). Samples BI-2 and BI-3 showed a high concentration of elements like lithium (Li) and zirconium (Zr) compared to the values of the samples from sites BII and BIV. Sample BII-2 presented a high concentration of minor elements such as rubidium (Rb), strontium (Sr), vanadium (V) and cerium (Ce). Finally, in sample BIV-2 barium (Ba) concentration and in sample BIV-3, lanthanium (La) and europium (Eu) concentrations were much higher than in the other samples.

Organic carbon content and pH measurements

The organic carbon content measured, on a dry weight basis, for the bentonite samples was very low in the range of 0.03-0.12% (Table 3). These values are much lower than those described for other clays. Clays contain, generally, organic carbon in the range of 0.1–5.0% (e.g. 0.6 ± 0.3% in Opalinus Clay [51], and 1–5% in Boom Clay [52]). The pH measured was 9.03, 7.82 [22] and 8.03, for superficial samples BI-2, BII-2 and BIV-2, respectively (Table 3). The pH value for sample BI-3 was 9.16 and for sample BIV-3 was 8.32.

Bacterial diversity analysis

Due to the differences observed in the RISA profiles (Online Resource 3), the five bentonite samples were further analysed to study the bacterial community. Therefore, two complementary techniques were applied to get a deep characterization, by semi-quantification of bacterial diversity using Illumina-sequencing and to get a precise taxonomical affiliation of the bacterial community by classical clone library analysis. NGS platform offers a high-throughput culture-independent analysis. After

normalization, a total of 13179 sequences per sample were annotated with a length of 244 nucleotides. A number of 174 Operational Taxonomic Units (OTUs) were discretely separated and classified into class (98% of phylotypes), order (96% of phylotypes), family (83% of phylotypes) and genus (51% of phylotypes) levels (Online Resource 4). In total, 174 phylotypes belonging to 13 different bacterial phyla (*Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Bacteroidetes*, *Choloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria, Nitrospirae, Verrucomicrobia* and an unknown bacterial phylum) were identified (Fig. 3). Rarefaction curves were plotted to evaluate the quality of the sampling (Fig. 2). As the curves reached a plateau, the sequencing for each sample was deep enough to detect all phylotypes. Richness, evenness and phylotype diversity were measured using conventional diversity indices (Table 4). These indices are based on species richness/evenness data from each sample. The resulting RFLP-predominant groups and RFLP-individuals of 100 clones per library were sequenced as follow: 19 predominant groups and 38 individual clones in sample BI-2; 8 predominant groups and 71 individual clones in sample BI-3; in the case of sample BII-2 and BIV-2, 14 and 57 individuals clones, respectively, and 13 dominant groups for each of both samples; finally, 12 predominant groups and 8 individual clones in sample BIV-3. After the sequencing, 14 bacterial phyla were taxonomically affiliated to *Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Bacteroidetes*, *Choloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria, Nitrospirae* and *Verrucomicrobia*, as well as to one unkown bacterial phylum (Fig. 4). Due to the similar results obtained by Illumina and cloning sequencing it is possible to compare the bacterial diversity data of both methods to get deeper information of the population of each bentonite sample.

The predominant phylum in sample BI-2 was *Bacteroidetes* (Fig. 3), represented by 48.9% of all sequences. The principal families semi-quantified were *Cytophagaceae* and *Chitinophagaceae*, as well as *Flaviobacteriaceae* (Online Resource 4). Belonging to family *Cytophagaceae* some clones were taxonomically identified up to genus, as for example, BI-2-62 affiliated to *Pontibacter* sp. MDT2-9. Clones belonging to family *Chitinophagaceae* were also identified: BI-2-2 affiliated to *Flavisolibacter ginsengisoli* strain Gsoil 643 [53], and BI-2-64 affiliated to *Flavisolibacter* sp. MDT2-37 (Online Resource 5). One of the dominant genera was *Flavobacterium*, OTU-77 (Online Resource 4), also detected as the individual clone BI-2-113, which affiliated to the uncultured *Flavobacterium* sp. clone bsc41. However, most of the *Bacteroidetes* clone sequences in sample BI-2 were affiliated to uncultured

Bacteroidetes bacteria (Online Resource 5). The second dominant phylum in sample BI-2 was *Proteobacteria* (26.2%) with affiliations to *Alpha*-, *Beta*-, *Delta*- and *Gammaproteobacteria* (Fig. 3 and 4). *Alphaproteobacteria* class was represented by 13% of the proteobacterial phylotypes by Illumina, mainly by genus *Sphingomonas* (15 OTUs). This genus was not one of the most abundant, since alphaproteobacterial clone sequences were taxonomically affiliated to other genera, such as *Porphyrobacter*, *Brevundimonas* and *Rhodobacter* (Online Resource 5). *Betaproteobacteria* class was mainly dominated by families *Comamonadaceae* and *Oxalobacteraceae* via Illumina. BI-2 clone sequences were mainly represented by family *Oxalobacteraceae* (*Herbaspirillum* spp. and *Massilia* sp.). Interestingly, one clone sequence belonged to phylum *Deinococcus*-*Thermus*, which was only found in sample BI-2. This represents a minor abundant phylum that could not be detected by Illumina sequencing. Slight differences were observed between the samples BI-2 (surface) and BI-3 (20 cm deep). Analysing the bacterial community of sample BI-3, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were the main phyla identified, represented by 42%, 26% and 12% of all the sequences, respectively (Fig. 3). *Beta*- and *Alphaproteobacteria* were the predominant *Proteobacteria* classes. In this sample, *Comamonadaceae* was the most significant betaproteobacterial family by Illumina (Online Resource 4), represented also by two identified clones affiliated to uncultured *Comamonadaceae* bacterium clone Ppss Ma80 (clone BI-3-27) and *Comamonadaceae* bacterium b4M (clone BI-3-102). Moreover, several clones belonging to family *Oxalobacteraceae* were detected, e.g. *Herbaspirillum* spp. (clones BI-3-24 and BI-3-46) and clone BI-3-100, whose sequence was affiliated to uncultured *Janthinobacterium* sp. clone cEIII43 (Online Resource 6). *Sphingomonas* (85%) was the predominant alphaproteobacterial genus via Illumina (Online Resource 4), as in the sample BI-2. In addition, the majority of the alphaproteobacterial clones belonged to genera *Blastomonas*, *Tabrizicola* and *Mesorhizobium* (Online Resource 6). Belonging to *Bacteroidetes*, *Cytophagaceae* was detected as the principal family (Online Resource 4), represented by genera *Pontibacter* (clones BI-3-55, BI-3-56 and BI-3-98) and *Rufibacter* (clone BI-3-51). Different *Flavisolibacter* species (BI-3-5, -43, -68 and -69) and uncultured *Bacteroidetes* bacteria (BI-3-1, -2, -29, -9, -59, -71, -72) were also affiliated to some clones of this sample (Online Resource 6). In the case of *Actinobacteria* phylum two different genera were dominant by Illumina, *Arthrobacter* and *Gaiella* (Online Resource 4). Moreover, some of the actinobacterial clones sequences were affiliated with *Arthrobacter* spp. (Online Resource 6). Superficial samples BI-2 and BIV-2 showed a higher diversity compared to the other samples, although the phyla distribution is different. While in sample BI-2

Bacteroidetes is dominant, in sample BIV-2, the overbearing phylum was *Proteobacteria* (52% of all sequences) with affiliations mainly to *Betaproteobacteria* (27%), followed by *Alpha-* (16%), *Delta-* (9%) and *Gammaproteobacteria* in very low proportion (Fig. 3). The prevailing betaproteobacterial order was *Burkholderiales*, the major families were *Comamonadaceae* (genus *Variovorax*: clones BIV-2-30, -31 and -66) and *Oxalobacteraceae* (genus *Herbaspirillum*: clones BIV-2-5, -23, -35 and -104) (Online Resource 7). Belonging to *Alpha-* and *Gammaproteobacteria*, genus *Sphingomonas* and *Xanthomonadaceae* family were dominant, respectively. Other abundant phylum in sample BIV-2 was *Bacteroidetes*. The principal families of this phylum were *Sphingobacteriaceae, Chitinophagaceae* and *Cytophagaceae* (Online Resource 4). On the genus level could be only *Hymenobacter* identified, which was also detected in the clone library (clone BIV-2-103). The majority of the *Bacteroidetes* clone sequences in sample BIV-2 were affiliated, as in the case of BI-2 and BI-3 samples (Online Resource 5 and 6), to uncultured *Bacteroidetes* bacteria, but also to *Sphingobacteriaceae* family (Online Resource 7). Additionally, clone representatives of phylum *Actinobacteria*, *Planctomycetes*, *Cyanobacteria* and *Gemmatimonadetes* were found in the sample BIV-2, which were also detected in both or just one of the BI samples. Taxonomical affiliation to *Actinobacteria* (one predominant group and twelve individual clones) was more abundant than to *Bacteroidetes* (two predominant groups and six individual clones) in sample BIV-2 (Online Resource 7). Finally, bacterial diversity in sample BII-2 was significantly different to those of the samples BI-2, BI-3, and BIV-2, but similar to BIV-3. Although samples BII-2 and BIV-3 were taken from different bentonite formations, they represented a similar bacterial community composition (Fig. 3 and 4). Indeed, the same predominant phylotypes of sample BII-2 were also prevalent in sample BIV-3 (Online Resource 4). The main class was *Betaproteobacteria* (98% and 83% of all the sequences, for BII-2 and BIV-3, respectively, as shown in Fig. 3). The betaproteobacterial clone sequences were affiliated with different genera, as shown in Fig. 5. In correlation with Online Resource 8, where it is observed that *Ralstonia* spp. and *Burkholderia* spp. were the most detected ones in both samples, the dominant phylotypes were genus *Ralstonia*, *Comamonadaceae* family and genus *Burkholderia*, in decreasing order (Online Resource 4). The genera *Pelomonas* and *Curvibacter* were, in contrast to the rest of the samples, only identified in samples BII-2 and BIV-3. Clone sequences belonging to genera *Hydrogenophaga* and *Acidovorax* were only detected in sample BII-2 (Online Resource 9) and clone sequences belonging to *Ramlibacter* only in sample BIV-3 (Online Resource 10). *Variovorax* spp. were detected by cloning in sample BII-2, but not in sample BIV-3 (Online Resource 8). Taxonomical affiliation to *Acidobacteria* was detected in sample BIV-

by cloning (Online Resource 10), but not to *Bacteroidetes*. However phylum *Bacteroidetes* was detected via Illumina sequencing. There were also some predominant OTUs in each of the five samples analyzed (Online Resource 4). In sample BI-2, OTU-22, -24 and -30, annotated as *Chitinophagaceae*, *Cytophagaceae* and *Flexibacter*, respectively, were highly enriched compared to the rest. In the case of sample BI-3, the most enriched OTUs were OTU-22, -26 and 42, affiliated, respectively, to *Chitinophagaceae*, *Comamonadaceae* and *Gemmatimonadaceae* families. *Ralstonia* (OTU-6), *Comamonadaceae* (OTU-13) and *Burkholderia* (OTU-14) were the highest enriched OTUs in both samples BII-2 and BIV-3. For sample BIV-2, the predominant OTUs were OTU-18 and -27, annotated as *Sphingobacteriaceae* and *Sphingomonas*, respectively.

Statistical multivariate analysis (principal component analysis, PCA)

PCA showed that the studied geochemical and mineralogical variables such as pH, TOC, etc. not contributing a major explanation to the bacterial diversity detected either by Illumina sequencing or traditional clone library analysis (Fig. 6). However, some samples showed light correlations to some geochemical parameters. For example, according to the bacterial diversity analysis, a correlation of samples BII-2 and BIV-3 with *Betaproteobacteria* class was found. Moreover, a correlation of those samples with Jarosite was also found. Curiously, these two samples showed the lowest diversity, dominated by *Betaproteobacteria*. Lower correlations were found for TOC and pH with sample BI-2.

Discussion

In this study, the structure and composition of bacterial populations in bentonite formations, considered as safety barriers (i.e., clay buffer and stone) within a future Spanish Deep Geological Repository (DGR), were analyzed by two different culture-independent assessments of 16S ribosomal RNA genes: classical clone libraries analysis and analysis of the V5–V6 hypervariable region through Illumina platform. Wouters and coworkers [7] suggested that the clear correlation between microbial diversity and TOC content in water samples collected from the underground Boom Clay Facility in Belgium is due to the bioavailability of the carbon source. In our case, there is no clear correlation between the bacterial diversity and TOC content determined in the studied samples. Sample BI-2 and BI-3 are the samples with the highest and the lowest TOC (Table 3), respectively. However, both samples present very similar

species richness (Table 4). On the other side there is no information about the bioavailability of the carbon sources available.

The same bacterial phyla were identified by both methods in this study, with the only exception of the detection of *Deinococcus-Thermus* by cloning, which revealed the good quality of both methods to identify the bacterial population of an environment. Interestingly, highly comparable affiliation of the population was found by Illumina sequencing ($16S_{807f}$ and $16S_{1050r}$) and by cloning ($16S\text{-}_{8f}$ and $16S\text{-}_{1492r}$). For example, *Proteobacteria* (mainly *Alpha*- and *Betaproteobacteria*), *Bacteroidetes*, *Actinobacteria* and *Acidobacteria* were the main bacterial phyla present in the studied bentonite samples. The structure and composition of populations in clays considered as host rock for deep geological disposal of radioactive wastes are poorly studied [21, 9]. The prevalence and abundance of particular phylotypes differed between the 3 superficial bentonite samples (BI-2, BII-2 and BIV-2). For example, *Bacteroidetes* (49% of total of phylotypes) and *Betaproteobacteria* class (98%) dominate the bacterial population of sample BI-2 and BII-2, respectively. In the case of sample BIV-2 bacterial dominance is divided among *Betaproteobacteria* class (27%) and *Bacteroidetes* phylum (26%). The variations in bacterial diversity observed between clay samples could likely be influenced by the differences in their mineralogical and geochemical properties (Fig. 6). Sample BII-2 differs in the chemical content (Table 1, 2 and Online Resource 2) in many aspects. For example, this sample is characterized by the presence of sanidine, jarosite and a high amount of Fe_2O_3 (11% of the total oxides content) in comparison to the other studied samples where the Fe oxides content range is between 2-5% (Table 2). This could be a reason for the low species richness in sample BII-2. Betaproteobacterial clones identified in the sample BII-2 belonged mainly to *Burkholderiales* specially *Ralstonia* spp., *Burkholderia* spp., *Variovorax* spp. as well as *Curvibacter* spp. and *Acidovorax* spp. (Online Resource 8 and 9). *Ralstonia* spp. are implicated in the biogeochemical cycle of Fe through oxidation of Fe(II) [54] including solubilization by siderophore production. The high tolerance to heavy metals and organic compounds of *Ralstonia* spp. was previously described [55-56]. For example, *Ralstonia metallidurans* (renamed as *Cupriavidus metallireducens*) is a model microorganism for studies of metal resistance [57-58]. It was also demonstrated that *Variovorax* species are resistant to several metals: Cu, Cd, Pb and Zn [59]. Moreover, arsenite-oxidising *Variovorax* strains were identified by Majumder *et al*. [60]. Similar betaproteobacterial sequences were also detected in sample BIV-3, which is also dominated by *Betaproteobacteria*. But in addition to these, betaproteobacterial sequences belonging to families *Comamonadaceae* and *Oxalobacteraceae* (e.g.

Massilia spp.) were also found in the samples BI-2, BI-3 and BIV-2 (Online Resource 4). *Betaproteobacteria* represented also a major part of the bacterial community in deep subsurface clay borehole water in the Boom Clay, Belgium [7].

However, in the case of *Bacteroidetes*, this phylum dominates the bacterial community of samples BI-2, BI-3 and BIV-2. It is interesting, that in BI-2 and BI-3 samples different *Bacteroidetes* genera were identified in contrast to sample BIV-2 and BIV-3, where only a few of them were detected. In BI-2 sample the genera *Flexibacter* (OTU-30), *Flavobacterium* (OTU-77), *Cytophaga* (OTU-62), as well as *Pontibacter* (OTU-115) were identified. In BIV-2 and BIV-3 samples were instead a higher proportion of *Hymenobacter* spp. (OTU-54, OTU-81, OTU-107) found compared to samples BI-2 and BI-3. *Alphaproteobacteria* were observed in almost all studied samples (just not in BII-2), but represented by different species belonging to order *Rhizobiales*, *Rhodobacterales* and *Sphingomonadales*. Representatives of this phylum were also found in other clay samples [6] and in deep subsurface clay borehole water [7].

Samples BI-2, BI-3 and BIV-2 showed overall a higher bacterial diversity, where *Gammaproteobacteria*, *Actinobacteria* and *Firmicutes* were detected in small quantities similar to bacterial phyla found in deep subsurface clay borehole water in the Boom Clay, Belgium [7]. The results of samples BI-2 and BI-3 (Online Resource 4, 5 and 6) demonstrate that even when sample BI-3 was taken 20 cm deeper than sample BI-2, the predominant population in both samples, consisted of representatives of two phyla, *Bacteroidetes* and *Proteobacteria.* Surprisingly, the bacterial diversity was very similar at phylum level in these two samples, with small variations (Fig. 3 and 4). Although, sample BI-3 contains a lower amount of TOC and was collected from a greater depth. In addition, *Chloroflexi*, *Armatimonadetes*, *Firmicutes* and unknown phyla were only detected in samples BI-2 and BI-3. In the case of *Firmicutes*, only clone BI-3-106 was identified as *Anoxybacillus* sp. SCSIO 15096 in sample BI-3 (Online Resource 6). Microbial diversity at iron-clay interfaces inside a deep argillite geological formation in France was dominated by *Firmicutes*, *Actinobacteria* and *Proteobacteria* [61]. Bacteria related to *Firmicutes* could be isolated from Opalinus clay in Mont Terri, Switzerland [16, 17], but did not dominated the bacterial community by using culture-independent approaches. Sporulation is a common ability within the *Firmicutes* [62], which could make them more resistant against extraction of DNA. The biogeochemical processes of microorganisms, either indigenous to the repository´s host rock or introduced during the

construction of a repository [10, 63], may affect the safety of this long-term deep geological disposal system. Microbial processes can affect the geochemistry of clays and also the mobility and transport of radionuclides from the repository to the geosphere. Three different clay-microbe interaction mechanisms were described: (i) structural Fe-clay mineral transformation (oxidation/reduction), (ii) alteration of mineral surfaces by the production of siderophores and small-organic acids, (iii) formation of biofilm in the clay mineral surface [10]. Moreover, the anaerobic canister corrosion might be due to the activity of sulfate-reducing bacteria of the compacted bentonite buffer surrounding the canister [12] that accelerate the corrosion of the canister or the over-pack materials [64]. However, no sulfate-reducing bacteria were detected in this study by the used methods. The largest portion of bacterial populations found in the clay samples BII-2 (98%), BIV-3 (83%), BIV-2 (27%) and BI-3 (19%) belongs to *Betaproteobacteria* class, whose members are able to affect the structure of the Fe-containing clay minerals through different processes. For example, through redox based transformation of Fe, in the case of *Ralstonia* spp. or *Acidovorax* spp. [65], production of siderophores by *Ralstonia* [66] and formation of biofilm on the surfaces of minerals (e.g. *Acidovorax*). *Ralstonia* was the main genus identified (almost 50% of the total clones) in BII-2 and BIV-3 samples. For example, in sample BII-2, the biggest group of clones identified belonged to *Ralstonia pickettii* strain QL-A6, which is a congener strain of *Ralstonia solanacearum*, a soil-isolated strain, described for their capacity to oxidize Fe(II) in smectite soil [67]. Another clone identified in sample BII-2 was affiliated to *Ralstonia mannitolilytica* strain JN201, isolated from potassic trachyte soils and interacting with silicate minerals. In sample BII-2 a predominant group of clones was closely related to *Acidovorax* and *Variovorax* genera, described for their ability to oxidize iron [68]. *Acidovorax* plays important roles in iron corrosion by biofilm formations in flowing environment [69]. Other betaproteobacterial clones identified in these clay samples were affiliated to genera including *Herbaspirillium*, *Janthinobacterium* and *Massilia*. Some of these bacteria, as for example different species of *Sphingomonas*, *Herbaspirillum* and *Massilia*, have also been isolated from sample BI-2 and BII-2 using culture dependent approaches [22]. Additionally, some isolates were affiliated to *Micrococcus* spp., *Arthrobacter* spp. and *Kocuria* spp., belonging to phylum *Actinobacteria*; *Pseudomonas*, and *Stenotrophomonas*, belonging to *Proteobacteria* phylum; *Bacillus simplex* strain Qtx-12, belonging to *Firmicutes* and in addition, a pigmented yeast strain related to *Rhodotorula mucilaginosa* was also recovered from these formations [22]. All the described microbe-clay interactions processes may affect

negatively the structure of clay minerals, affecting the function of clay as a barrier by loosing swelling capacity, and enhance the risk of radionuclide mobilization.

Conclusions

The current work describes the bacterial diversity of Spanish bentonite formations, by 16S rRNA gene-based analysis via Illumina sequencing and traditional clone libraries. Results revealed a high bacterial diversity in bentonite samples BI-2, BI-3 and BIV-2, dominated by phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*. A clear dominance of *β-proteobacteria* class was detected in samples BII-2 and BIV-3. The majority of the bacterial OTUs and clones identified are strict or facultative aerobic microorganisms. Some of them are affiliated to bacterial strains described for their ability to affect the biogeochemical cycle of Fe by different processes or to interact efficiently with radionuclides. Hence, the effect of this bacterial community might play a role in the safety concept of the deep geological repository of radioactive wastes. Further analyses are required to get a deeper knowledge about the bacterial activity at repository relevant conditions and about the interaction mechanisms of the indigenous microorganisms with radionuclides.

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