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Microbiology of Barrier Component Analogues of a Deep Geological Repository

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Keywords: bentonite clay, rock core, Opalinus Clay, DNA extraction, 16S rRNA gene, sequencing, cultivation

Abstract

Canada is currently implementing a site selection process to identify a location for a deep geological repository (DGR) for the long-term storage of Canada's used nuclear fuel, wherein used nuclear fuel bundles will be sealed inside copper-coated carbon steel containers, encased in highly compacted bentonite clay buffer boxes and sealed deep underground in a stable geosphere. Because a DGR must remain functional for a million years, there is value to examining ancient natural systems that serve as analogues for planned DGR components. Specifically, studying the microbiology of natural analogue components of a DGR is important for developing an understanding of the types of microorganisms that may be able to grow and influence the long-term stability of a DGR. This study explored the abundance, viability, and composition of microorganisms in several ancient natural analogues using a combination of cultivation and cultivation-independent approaches. Samples were obtained from the Tsukinuno bentonite deposit (Japan) that formed ~10 mya, the Opalinus Clay formation (Switzerland) that formed ~174 mya, and Canadian shield crystalline rock from Northern Ontario that formed ~2.7 bya. Analysis of 16S rRNA gene amplicons revealed that three of the ten Tsukinuno bentonite samples analyzed were dominated by putative aerobic heterotrophs and fermenting bacteria from the *Actinobacteria* phylum, whereas five of the Tsukinuno bentonite samples were dominated by sequences associated with putative acidophilic chemolithoautotrophs capable of sulfur reduction. The remaining Tsukinuno bentonite samples, the Northern Ontario rock, and Opalinus Clay samples generated inconsistent replicate 16S rRNA gene profiles and were associated primarily with contaminant sequences, suggesting the microbial profiles detected were not sample-specific but spurious. Culturable aerobic heterotroph abundances were relatively low for all Tsukinuno bentonite samples and culturable anaerobic heterotrophs were only detected in half of the

Tsukinuno samples. Sulfate-reducing bacteria (SRB) were only detected in one Tsukinuno sample by cultivation. Culture-specific 16S rRNA gene profiles from Tsukinuno clay samples demonstrated the presence of phyla *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Firmicutes* among aerobic heterotroph cultures and additional bacteria from the phyla *Actinobacteria* and *Firmicutes* from anaerobic heterotroph plate incubations. Only one nucleic acid sequence detected from a culture was also associated with its corresponding clay sample profile, suggesting that nucleic acids from culturable bacteria were relatively rare within the clay samples. Sequencing of DNA extracted from the SRB culture revealed that the taxon present in the culture was affiliated with the *Desulfosporosinus* genus, which has been found in related bentonite clay analyses. Although the crystalline rock and Opalinus Clay samples were associated with inconsistent, likely spurious 16S rRNA gene profiles, we show evidence for viable and detectable microorganisms within several Tsukinuno natural analogue bentonite samples.

Key words: bentonite clay, rock core, Opalinus clay, DNA extraction, 16S rRNA gene sequencing, enrichment culture

Importance

Studying the microbiology of natural analogue components of a deep geological repository (DGR) for nuclear waste is important for developing an understanding of the types of microorganisms that may be able to grow and influence the long-term stability of a DGR. This is one of few studies that uses DNA sequencing approaches to study natural bentonite samples, and the first to directly compare microbial profiles generated from direct DNA extraction from

natural bentonite clay to those generated from DNA extraction from cultures obtained from the same samples. Because microbiology is critical for developing a safety case for DGR design, the study of natural analogue samples will help inform design considerations based on anticipated microbial communities associated with clay and rock over geological time.

Introduction

In Canada and around the world, nuclear reactors are powered by solid uranium dioxide fuel pellets housed in fuel bundles (Satyanarayana et al. 2018). After a fuel bundle has served its purpose in a nuclear reactor and is removed, it is highly radioactive and is typically stored on an interim basis and monitored at the reactor site. While the used fuel radioactivity declines with time it takes many millennia for the used fuel radioactivity to decrease to a benign level and therefore a more permanent solution is required (Weber et al. 2009). To ensure the indefinite isolation of used nuclear fuel from the environment, Canada, along with many other countries, is using the deep geological repository (DGR) concept as the long-term storage solution for used nuclear fuel. While DGR design concepts vary internationally, all DGR designs incorporate a multibarrier system to ensure the long-term isolation of the used fuel from the environment (Hall et al. 2020). Canada's DGR concept and multibarrier design are demonstrated in Supplemental Figure 1. The first two barriers, the used fuel pellets (highly stable ceramic) and zircaloy fuel cladding (corrosion resistant metal) which comprise the used fuel bundle are particularly stable in the highly reducing ground water that will be present at repository depth. These used fuel bundles will be sealed into the third barrier, a long-lived used fuel container (UFC). The UFCs are made of structurally stable carbon steel and coated with copper for corrosion resistance to prevent exposure of the fuel bundles to ground water for hundreds of thousands of years (Hall et

al. 2020). Next, UFCs will be encased in buffer boxes made of highly compacted bentonite clay, a swelling smectite clay, and placed in the underground (Supplemental Figure 1). As the placement of bentonite buffer boxes in the DGR progresses, void spaces between the rock wall and the buffer boxes will be filled in with a granulated bentonite clay (King et al. 2017). The final barrier is the stable geosphere in which the DGR is constructed approximately 500-600 m below ground.

In addition to planning for multiple natural and engineered barriers, there are several factors that must be considered to ensure that the integrity of a DGR and its components are stable over very long timeframes. One such factor is the role that microorganisms within or surrounding the DGR could play. In general, DGRs are predicted to be relatively inhospitable environments for microbial activity as a result of limited water availability, lack of space and increased mechanical force due to the highly compacted nature of saturated bentonite clay, low nutrient availability, high temperatures, and a radiation field surrounding the UFCs (King 2009). Nonetheless, early studies using cultivation-based techniques demonstrated that microorganisms are naturally found in deep subsurface environments (Balkwill et al. 1989, Kieft et al. 1997) and bentonite (Masurat et al. 2010), thus necessitating the need to study their potential viability and activity within the context of a DGR emplacement.

The construction of a DGR will impact the natural deep subsurface environment and its naturally occurring microbial communities through the introduction of oxygen, water, space, nutrients, and exogenous microorganisms, which may initially favour growth of aerobes and facultative anaerobes (Leupin et al. 2017). Once oxygen and nutrients have been consumed, the DGR will

return to reducing conditions. In the absence of oxygen, light, and readily available organic matter, chemolithoautotrophic forms of primary production may be favoured. Many types of metabolisms may be possible in a DGR environment (Supplemental Figure 2). Specifically, the potential production of hydrogen sulfide or gases are of particular importance due to their potential to have a direct impact on components of the multibarrier system. Hydrogen sulfide (H_2S) production is an important consideration as this metabolic end product can corrode metals, including the copper and steel of UFCs, through a process termed microbiologically influenced corrosion (MIC). Due to the ubiquitous nature of sulfate (SO_4^{2-}), sulfate-reducing bacteria (SRB) tend to dominate instances of MIC within anoxic environments (Wu et al. 2016, Bagnoud et al. 2016, Bell et al. 2018, 2020, Boylan et al. 2019, Gu et al. 2019) and can also proliferate within SO_4^{2-} -limited systems (Purkamo et al. 2014, 2016, Hernsdorf et al. 2017). Additionally, SRB have been detected within commercially available bentonites proposed for use in DGR construction (Masurat et al. 2010).

Early microbiological analysis of bentonite clays focused on cultivation methods to identify and quantify detectable microorganisms (Motamedi et al. 1996, Kieft et al. 1997, Pedersen et al. 2000, Mauclaire et al. 2007, Poulain et al. 2008, Stroes-Gascoyne et al. 2010, Urios et al. 2012, López-Fernández et al. 2014). Several studies only considered the culturability of SRB due to their relevance to DGR safety (Motamedi et al. 1996), whereas others also considered aerobic and anaerobic heterotrophs (Mauclaire et al. 2007, Poulain et al. 2008, Stroes-Gascoyne et al. 2010, Urios et al. 2012, López-Fernández et al. 2014). These studies often involved microcosm-style experiments with bentonite clay incubated under DGR relevant conditions, and have led to DGR design elements such as maintaining a bentonite water activity of greater than 0.96 to

suppress microbial growth (Stroes-Gascoyne et al. 2010). Although these methods can give insight into the types of microorganisms that could be active under DGR conditions, and the conditions that could suppress their growth, only microorganisms that can grow in the given cultivation conditions are considered. Depending on the cultivation conditions applied, this could mean that only rare microorganisms, or microorganisms with less relevant metabolisms to the evolution of the DGR are targeted. More recent studies on the microbiology of bentonite clay have made use of DNA sequencing methods to overcome some of the limitations of cultivation approaches. The main obstacle faced when using DNA-based methods for the microbial characterization of clay is the difficulty of efficiently extracting sufficient DNA for detection due to the low biomass nature of clay combined with its adsorptive properties. The use of DNA extraction and subsequent sequencing for clay has only been reported in a few cases (Boivin-Jahns et al. 1996, Lopez-Fernandez et al. 2015, Engel et al. 2019b, Bolyen et al. 2019, Povedano-Priego et al. 2021), including the study in which the DNA extraction method used in the current study was validated (Engel et al. 2019a). Although several groups have explored bentonite clay microbiology under DGR-relevant microcosm experiments (Motamedi et al. 1996, Kieft et al. 1997, Pedersen et al. 2000, Stroes-Gascoyne et al. 2010, Jalique et al. 2016, Povedano-Priego et al. 2021), very few studies have looked at the microbiology of naturally occurring ancient clays and rocks that may serve directly as analogues for future DGR barrier components. Even fewer studies have employed sequencing techniques to analyze these DGR barrier component analogues using a combination of cultivation and sequencing techniques for the same samples (López-Fernández et al. 2014, Lopez-Fernandez et al. 2015).

The purpose of the current study was to analyze the microbiology of ancient natural analogues of DGR barrier components using a combination of cultivation and cultivation-independent approaches. The specific goals of this research were to estimate the number of microorganisms present, assess the viability of microbial groups using cultivation techniques, quantify DNA templates (from cells and relic DNA) using quantitative polymerase chain reaction (qPCR), and generate microbial profiles using 16S rRNA genes to assess *in situ* nucleic acids from these analogue samples.

Three different ancient natural barrier component analogues were selected for microbiological analysis. Ten ancient bentonite samples were taken from the Tsukinuno bentonite deposit, which hosts the largest underground bentonite mine in Japan, consisting of 35 layers of bentonite within the mining area (Supplemental Figure 3, Table 1) (Yokoyama et al. 2004). This bentonite deposit was formed in the middle Miocene (16-11.7 million years ago) and is composed primarily of sodium smectite with smaller amounts of quartz, feldspar, illite, calcite, and zeolite. Although the bentonite samples were taken from a tunnel wall exposed to oxygen instead of cores taken from a depth, the analysis of these samples can serve as an analogue to bentonite in a DGR during the initial oxic phase. The ten samples were taken from five different bentonite clay beds, with samples being characterized by different texture, colour, and moisture level (Table 1).

Additionally, one sample was taken from an Opalinus Clay formation, which is the only host rock currently being considered for the placement of Switzerland's DGR (Supplemental Figure 4). Opalinus Clay is a Mesozoic shale formation that was formed about 174 million years ago in the Jura Mountains in northwestern Switzerland (Bleyen et al. 2017). Opalinus Clay consists of approximately 65% clay minerals, namely illite, smectite, kaolinite, and chlorite, of which 10%

are swelling clays (Thury and Bossart 1999, Poulain et al. 2008). Finally, three rock cores were taken from a crystalline rock formation in Northern Ontario (Canada; Supplemental Figure 4).

The Northern Ontario crystalline rock (NOCR) sample site is situated in the Superior province of the Canadian shield, large portions of which have been stable for the last 2.5 billion years.

Materials and methods

Sampling and storage

Bentonite clay was sampled opportunistically from ten locations along a mining tunnel wall in the Tsukinuno mine in Japan as a part of the International Bentonite Longevity (IBL) project. The tunnel surface was scraped to a depth of approximately 20 cm before samples were taken using steel tools. Clay samples were stored in plastic bags throughout the sampling process. The samples were then packaged in plastic wrap and aluminum foil before shipping, and were stored at 4°C upon arrival at the University of Waterloo. After subsamples were taken for analyses, the remaining bentonite was stored at -20°C. A 10 cm core of Opalinus Clay (O1) was drilled in 2017 using non-sterile pressurized air. The core was placed in sterile bags and air was evacuated. The core was stored on dry ice until arrival at the University of Waterloo, at which point it was stored at -80°C until processing. For Northern Ontario crystalline rock (NOCR) samples, a borehole was drilled in 2017 and cores were recovered. Sample N2 came from a depth of 99.0-99.3 m, sample N4 came from a depth of 219.7-219.9 m, and sample N6 came from a depth of 513.4-513.7 m belowground. The outer layer of each core was sampled using sterile swabs. Both swabs and rock samples were stored at -20°C until processing.

Sample preparation

The outer layers of bentonite and Opalinus Clay samples were removed using a sterile scalpel to eliminate possible contaminants introduced during the sampling process. The inner layers of the Tsukinuno bentonite samples were crushed using a mortar and pestle to achieve a homogeneous mixture of the clay. The inner layer of the Opalinus Clay was crushed in a 50 mL stainless steel grinding jar (Retsch) in a MM 400 mixer mill (Retsch). The mortars, pestles, and grinding jars were rinsed with bleach (2.5% sodium hypochlorite), followed by Milli-Q water, before usage and were swabbed to identify any contaminants introduced during the sample crushing process. Crushing of the NOCR cores was performed by personnel at McMaster University. The three cores were cut into slices and the outer layer was removed using a hammer and chisel that were rinsed with dichloromethane, acetone, then methanol. The inner samples were crushed using a stainless-steel mortar and pestle, and then ground to a 1 mm grain size using a puck mill. Crushed inner layer samples and outer layer swabs were sent to the University of Waterloo where they were stored at -80°C until further processing.

Water activity and moisture content

A WP4C Water Potential Meter (Decagon) was used to measure water activity of the Tsukinuno bentonite clay samples using the fast mode of the instrument. From a separate aliquot of each sample, weights were recorded before oven baking at 110°C for 48 hours. The difference in weight before and after baking was used to determine the moisture content of the sample. These values were used to report measurements of 16S rRNA gene copy numbers, most probable number (MPN), and colony forming unit (CFU) on a per gram dry weight basis. Water activity and moisture content of the Opalinus Clay and NOCR samples were not measured because these

samples were stored in a -80 °C freezer for over two years before processing for this project, and measurements would thus be expected to be unreliable due to both storage time and temperature.

Cultivation

Two grams of the crushed Tsukinuno bentonite clay samples were suspended in 18 mL of phosphate buffered saline (PBS) solution (dilution A) and vortexed at low speed for 30 minutes. Two 10-fold serial dilutions were made from the original clay suspension (dilutions B and C) and these were used to inoculate aerobic and anaerobic heterotroph and SRB cultures as described below.

Dilution A (100 µL) was added to six Reasoner's 2A (R2A; M1687, HiMedia Laboratories, PA, USA) agar plates and dilution B (100 µL) was added to three R2A agar plates. The liquid was spread onto the surface of the agar with a flame-sterilized glass spreader until the surface of the agar was dry. Three of the dilution A plates and all of the dilution B plates were incubated under oxic conditions in a 30°C incubator for seven days. The remaining three dilution A plates were placed in an anaerobic chamber (Best Value Vacs, three gallon stainless steel vacuum chamber) containing a GasPak EZ anaerobe sachet (BD), which was evacuated and flushed three times with a 90% N₂ and 10% CO₂ gas mixture to create an anoxic environment. The chamber was placed in a 30°C incubator for 28 days. After incubation, colonies were counted on each of the plates, and the number of CFU per gram of clay was calculated.

One mL of dilution A, B, and C were also added to five test tubes, each containing 9 mL of an SRB enrichment medium (M803, HiMedia Laboratories, PA, USA), and tubes were placed

in anaerobic chambers that were evacuated and flushed as described above. Chambers were placed in a 30°C incubator for 28 days. After incubation, tubes positive for SRB activity were identified by the formation of a black ferrous sulfide precipitate. The MPN method was used to estimate the number of SRB present in one gram of the original clay sample.

Cultivation approaches were not used for the Opalinus Clay and NOCR samples because these samples were stored in a -80 °C freezer for over two years before processing and measurements would thus be unreliable.

DNA extraction

Total genomic DNA was extracted from the Tsukinuno bentonite clay, Opalinus Clay, and NOCR samples, from the aerobic heterotroph, anaerobic heterotroph, and SRB cultures, and from the mortar and pestle controls. A kit control containing no sample was included for each batch of extractions. The DNA extraction method used for clay and rock samples was validated previously (Engel et al. 2019a). Briefly, total genomic DNA was extracted from duplicate 5-g aliquots of the crushed inner layer of each clay sample and 10-g aliquots of the crushed inner layer of each rock sample using the DNeasy PowerMax Kit (Qiagen). After addition of lysis solution, PowerBead tubes were incubated at 65°C for 30 minutes, followed by bead beating at 30 Hz for 10 minutes using a MM 400 mixer mill. The remainder of the extraction was carried out following the manufacturer's instructions. Extracted DNA was eluted in 2 mL of elution buffer, quantified using the Qubit HS, and stored in aliquots at -20°C until further analysis.

A pipette tip was used to collect approximately equal amounts of microbial biomass from each colony type on the surface of the R2A agar plates, and the biomass was added to a separate microcentrifuge tube containing 20 μL of PCR water for anaerobic heterotrophs and aerobic heterotrophs of each of the ten Tsukinuno bentonite clay samples. A 1-mL aliquot of the positive SRB culture was centrifuged for 2 min at $10,000 \times g$ to form a cell pellet and the supernatant was decanted. Total genomic DNA was extracted from the cells of each culture type for each sample using the DNeasy UltraClean Microbial Kit (Qiagen). After addition of lysis solution, the PowerBead tubes were incubated at 65°C for 10 min, followed by bead beating using a FastPrep instrument (MP Biomedicals, OH, USA) at 5.5 m/s for 45 s. The remainder of the extraction was carried out following the manufacturer's instructions. Extracted DNA was eluted in 50 μL of elution buffer and stored in aliquots at -20°C until PCR analysis.

All DNA samples were quantified using the Qubit dsDNA High Sensitivity Assay kit (Invitrogen, CA, USA, cat. No. Q32854). Ten μL of DNA extract was added to 190 μL of Qubit dsDNA HS reagent diluted 200-fold in buffer. The mixture was vortexed, and fluorescence was measured using a Qubit 4.0 fluorometer (Life Technologies, CA, USA) after three minutes of incubation (Table 1).

Quantitative PCR

Bacterial 16S rRNA genes were quantified using qPCR with primers 341F and 518R (Muyzer *et al.*, 1993). The 15 μL PCR mix contained $1 \times$ SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.3 μM final concentration of each primer, 7.5 μg of bovine serum albumin (BSA), and 4 μL of template DNA (between 0 and 1 ng; see concentrations of DNA from Tsukinuno

bentonite clay samples included in Table 1). The PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) as follows: 98°C for three minutes followed by 40 cycles of 98°C for 15 seconds and 55°C for 30 seconds. The V3 to V5 region of the *Thermus thermophilus* 16S rRNA gene was previously cloned into the pUC57-Kan plasmid, flanked by M13F/R primers. Both primers were used to amplify the 16S rRNA gene fragment, which was then purified and used as a template for the standard curve. Bacterial 16S rRNA gene copy numbers were calculated from linear regression of the standard curve.

Amplification and sequencing of 16S rRNA genes

The V4-V5 region of the 16S rRNA genes were amplified in triplicate using universal primers 515F-Y (Parada et al. 2016) and 926R (Quince et al. 2011), modified to contain a unique 6 base index sequence and flow cell binding and sequencing sites (Bartram et al. 2011). The PCR was prepared in a workstation with ISO 5 HEPA-filtered air and surfaces treated with UV light (AirClean Systems, ON, Canada). The tubes, PCR water, and bovine serum albumin (BSA) were UV-treated for 20 minutes with 302 nm wavelength light on a transilluminator (ProteinSimple, CA, USA). The 25 µL PCR mix contained 1× ThermoPol Buffer, 0.2 µM final concentrations of forward and reverse primer, 200 µM final concentration of dNTPs, 15 µg BSA, 0.625 units hot start *Taq* DNA polymerase (New England Biolabs, MA, USA), and 1 µL of template DNA (between 0 and 0.3 ng). The PCR was performed as follows: 95°C initial denaturation for three minutes, followed by 35, 40, 45, or 50 cycles of 95°C denaturation for 30 sec, 50°C annealing for 30 sec, 68°C extension for 1 min, and a final extension of 68°C for 7 min. Each biological replicate of a clay or rock samples was amplified for 40, 45, and 50 cycles to identify the impact of cycle number on amplification product and subsequent sequence data. Thus, each sample had two biological replicates and three technical “cycle number replicates” for each (a

total of six replicates for each sample). The lowest biomass samples had insufficient amplification from certain replicates for there to be remaining reads after sequencing and quality filtration, so several samples have fewer than six replicates. The term “replicate” is used throughout to refer to the combination of biological and technical cycle number replicates of a sample. DNA extracted from cultures was amplified for 35 cycles. Negative PCR controls containing no template DNA were included in the 96-well plates to test for cross-contamination effects and were also prepared in single tubes to test for background amplification from the PCR mixture. Triplicate, uniquely indexed PCR products were pooled and quantified on a 1% agarose gel containing GelRed (Biotium, CA, USA), and equimolar quantities of each amplicon were pooled into a single tube. Negative PCR controls, and PCR-amplified DNA extraction kit controls and swab controls (unused swabs) were included in the sequencing pool (5 μ L each), even if amplification was not detected on the gel. The pooled 16S rRNA gene amplicons were run on a 1.5% agarose gel and were then excised and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA). The library was denatured and diluted following Illumina guidelines (Document no. 15039740 v01). An 8-pM library containing 15% PhiX control v3 (Illumina Canada Inc, NB, Canada) was sequenced on a MiSeq instrument (Illumina, CA, USA) using a 2×250 cycle MiSeq Reagent Kit v2 (Illumina Canada).

The 16S rRNA gene amplicons from all Tsukinuno bentonite clay, Opalinus Clay, NOCR, and SRB and anaerobic heterotroph cultures were sequenced in a single run with a cluster density of 799 ± 65 , and 89% of clusters passing the quality filter. The extracted DNA from the aerobic heterotroph cultures was sequenced in a second sequencing run with a cluster density of 775 ± 78 and 90% passing filter.

Sequence analysis

Sequence reads were demultiplexed using MiSeq Reporter software version 2.5.0.5 (Illumina) and analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2, release 2019-10) (Bolyen et al. 2019). Paired end reads were imported into QIIME2 using *qiime tools import*. DADA2 (*dada2 denoise-paired*) (Callahan et al. 2016) was used to remove low quality reads and primer sequences, and to truncate the forward and reverse reads to 250 bases. Furthermore, DADA2 was used to denoise and dereplicate paired-end reads, and generate ASVs. Taxonomy was assigned to ASVs using a naive Bayes classifier (*feature-classifier classify-sklearn*) pre-trained with SILVA database release 132 (Pruesse et al. 2007). These analyses were managed by Automation, Extension, and Integration Of Microbial Ecology, version 3 (AXIOME3; Min et al, 2021).

Contaminant ASVs were identified using the Decontam R package (Davis et al. 2018) with a threshold value of 0.5. The threshold value was selected by analyzing histograms of score statistic distribution across all samples (Supplemental Figure 8) and bubble plots showing the identity of putative contaminant ASVs at all possible threshold values. Due to the different levels of contaminant sequences detected among samples PCR-amplified with different numbers of cycles, the clay and rock samples amplified with 40, 45, and 50 cycles of PCR were all processed with Decontam separately. Note that although Decontam does remove certain ASVs found in both negative controls and samples, it does not result in the removal of all ASVs present in both controls and samples. Especially for low biomass, contaminant-dominated samples, further careful analysis of 16S rRNA gene profiles often identifies additional ASVs as likely contaminants. After contaminant identification, contaminant ASVs were removed from the ASV

tables Supplemental Figure 9-11). These ASV tables were used for all subsequent analyses. Predictions on the types of metabolisms possible within the microbial community were made from this ASV table using FAPROTAX (Louca et al. 2016). A triplot was made from the ASV table using AXIOME3 (Min et al. 2021).

Statistical tests (Spearman correlation, Pearson correlation, Wilcoxon signed-rank test, and Mann-Whitney U test) were run in R. The Spearman correlation was used for all correlations involving water activity (which does not follow a linear relationship), and Pearson correlation was used in all other cases. The Shapiro-Wilk test for normality was used to test if the distribution of samples was normal before selecting a test. As none of the sample groups tested were normally distributed, the Wilcoxon signed-rank test and Mann-Whitney U test were selected instead of their parametric T-test equivalents. Spearman correlation (r_s) was used for all correlations involving water activity (which does not follow a linear relationship), and Pearson correlation (r) was used in all other cases.

All sequences generated in this study were deposited in the European Nucleotide Archive with project accession number PRJEB39438.

Results and Discussion

Microbial abundance

An important objective of this research was to characterize microorganisms in ancient natural barrier component analogues: Tsukinuno bentonite clay, Opalinus clay, and Northern Ontario crystalline rock (NOCR) cores. Abundances of 16S rRNA genes, aerobic heterotrophs, anaerobic

heterotrophs, and SRB per gram of dry bentonite were measured for each of the Tsukinuno bentonite samples (Figure 1). The Tsukinuno bentonite samples had between 3.2×10^1 (T6) and 7.2×10^7 (T2) 16S rRNA gene copies/gdw. The number of 16S rRNA gene copies detected is likely an overestimate of the number of cells present, because most bacterial cells have multiple copies of the 16S rRNA gene and qPCR will also amplify relic DNA in addition to DNA from living cells (Acinas et al. 2004).

Aerobic heterotrophs were cultured from all Tsukinuno bentonite samples, with estimates of CFU/gdw between 4.1×10^1 (T9) and 1.1×10^4 (T2) (Figure 1). The average number of aerobic heterotrophs detected across the ten Tsukinuno bentonite samples was higher than the average number of anaerobic heterotrophs detected (Wilcoxon signed-rank test; $p < 0.05$). The Tsukinuno bentonite samples were taken from a tunnel wall and thus were all exposed to oxic conditions. As such, aerobes were expected to be in higher abundance than anaerobes. Anaerobic heterotrophs were only culturable from five samples (T2, T4, T6, T7, and T9) and SRB were only detected in one sample (T2). The culturability of anaerobic heterotrophs and SRB from these samples could be in part due to their increased water activity and moisture content compared to the drier samples (T3 and T10), from which anaerobic heterotrophs and SRB were not cultured, and culturable aerobic heterotrophs were less abundant (Supplemental Figure 5). Measures of CFU/gdw of anaerobic heterotrophs were between 4.5×10^1 (T4) and 9.9×10^2 (T7) across the five samples from where they were detected. Aerobic and anaerobic heterotrophs were previously cultured from bentonite cores taken from the Tsukinuno mine using similar methods to the ones used in this study (Fukunaga et al. 2005). They reported aerobic heterotroph estimates between 10^2 (identified as the lower limit of detection) and 10^6 CFU/gdw, and

anaerobic heterotrophs between 10^2 and 10^4 CFU/gdw, with decreasing counts with increasing distance from the drilling mouth (Fukunaga et al. 2005). The culture-based cell abundance estimates in the current study are within the range of these previous estimates.

Sample T2, the only sample where SRB were detected using the cultivation approach, had an MPN estimate of 5 SRB/gdw. The abundance of cultivatable SRB is likely lower in these clay samples than it would be in similar samples exposed to anoxic conditions because SRB are strict anaerobes, although previous studies have shown their ability to survive oxygen exposure for limited times (Kjeldsen et al. 2004, Dolla et al. 2006).

The Tsukinuno bentonite samples had a higher average number of 16S rRNA gene copies/gdw than the NOCR samples (Mann-Whitney U test; $p < 0.05$; Figure 1). Compared to the average measured 16S rRNA gene copies/gdw of $5.7 \times 10^6 \pm 2.3 \times 10^5$ for the ten Tsukinuno bentonite samples, the three NOCR samples had an average of $1.1 \times 10^3 \pm 4.7 \times 10^1$ 16S rRNA gene copies/g, and the Opalinus Clay sample had $1.2 \times 10^3 \pm 5.0 \times 10^2$ 16S rRNA genes copies/g.

In previous research quantifying the 16S rRNA gene copies in compacted and dry MX-80 bentonite clay, medians of 4.1×10^4 copies/g (Persson et al. 2011) and 1.7×10^7 copies/gdw (Engel et al. 2019a) were detected, respectively. Four of the ten Tsukinuno bentonite samples had 16S rRNA gene copies/gdw within the range of 10^4 and 10^7 , one of the samples was slightly higher than this range, and the remaining four were lower (Figure 1). The wide range of 16S rRNA gene copies/gdw measured in the Tsukinuno bentonite samples may be explained, at least in part, due to the broad range of water activities and moisture contents measured in these

samples (Supplemental Figure 5), which may have impacted bacterial growth or DNA extraction efficiency. The 16S rRNA gene copy number and moisture content of the Tsukinuno bentonite samples were strongly correlated ($r=0.72$, $p=0.02$), as was 16S rRNA gene copy number and water activity ($r_s=0.77$, $p=0.01$, Figure 2). The four samples with the highest 16S rRNA gene copy number were also the samples with highest water activities, and the only samples with water activities greater than 0.96, below which microbial activity has been shown to be suppressed in bentonite clay (Stroes-Gascoyne et al. 2010).

The 16S rRNA gene copy numbers of the Opalinus Clay and NOCR samples were all lower than the range of previously measured 16S rRNA gene copy numbers in manufactured MX-80 bentonite (Persson et al. 2011, Engel et al. 2019a). The number of 16S rRNA gene copies detected in the NOCR samples are more similar to the estimated microbial abundances of clay-dominated aquifer layers of 10^3 cells/gdw (Balkwill et al. 1989). The estimated microbial abundance of both the NOCR and Opalinus clay samples was orders of magnitude lower than the estimate of microbial abundance made for volcanic rock from Yucca mountain, a proposed host rock for an American DGR, with abundances reported between 3.2×10^4 and 3.2×10^5 cells/gdw (Kieft et al. 1997).

Microbial characterization of Tsukinuno bentonite DNA extracts

In ordination space, the replicates of all Tsukinuno bentonite samples grouped with each other, regardless of cycle number (Figure 3). A total of nine phyla were identified across all Tsukinuno bentonite samples at relative abundances equal to or greater than 5% (*Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Euryarchaeota*, *Firmicutes*, *Nitrospirae*,

Proteobacteria, and *Tenericutes*), with a representative of the *Euryarchaeota* associated with sample T4 in particular. This was the only member of an archaeal phylum detected in any sample with this relative abundance threshold. With a goodness of fit statistic (R^2) threshold of 0.2, water activity, DNA concentration, and moisture content correlated with samples within ordination space, with moisture content having the highest association (Figure 3). This indicates that these three factors are all associated with differences in microbial communities detected among samples.

Tsukinuno bentonite samples T1, T2, T4, T7, T8, and T9 each had replicates with consistent 16S rRNA gene profiles (Figure 4). For each of these samples, the 16S rRNA gene profiles were dominated (>50% relative abundance; Figure 4) by one to three amplicon sequence variants (ASVs). None of these dominant ASVs occurred in more than one sample. However, the 16S rRNA gene profiles show that samples taken from adjacent locations in the same bed had taxonomically similar dominant ASVs when the samples had similar appearance, water activities, and moisture contents. Although samples T3, T5, T6, and T10 had several shared ASVs among replicates, the profiles show a higher degree of variation. Overall, bed two (T3) had ASV representation from seven of the nine phyla detected across all samples at or above 5% abundance, bed 17 (T2) was dominated by *Proteobacteria*, bed 19 (T1; T5-T9) by *Actinobacteria* and *Proteobacteria*, bed 20 (T4) by *Euryarchaeota*, and bed 29 (T10) by *Actinobacteria*. The 16S rRNA gene analysis did not reveal the presence of any known SRB in any of the Tsukinuno bentonite samples.

Samples T3 and T6 showed the least consistent 16S rRNA gene profiles between

replicates, and the ASVs common to more than one replicate were associated with the genera *Staphylococcus* and *Afipia*, respectively. Although *Staphylococcus* is generally a mammalian skin-associated genus of bacteria, it has been cultured from bentonite clay in Spanish bentonite formations (López-Fernández et al. 2014), and from the Boom clay formation in two separate studies (Boivin-Jahns et al. 1996, Wouters et al. 2013); however, it was suggested in both to be a potential contaminant. All species within the genus *Afipia* are considered as opportunistic human pathogens (Brenner et al. 1991). The same ASV associated with *Afipia* was found at low relative abundance in all Tsukinuno bentonite samples with the exception T2, as well as several negative controls where it was sometimes a dominant member of the control microbial community. The combined inconsistency between replicate 16S rRNA gene profiles and the classification of the most prevalent ASVs in these samples as human-associated microorganisms strongly suggests that the 16S rRNA gene profiles of samples T3 and T6 do not represent true clay microbial community members but rather contamination from reagents or processing, or due to samples being taken from an exposed tunnel wall.

The two samples taken from locations adjacent to sample T6, samples T1 and T7, as well as sample T10, all had 16S rRNA gene profiles dominated by ASVs assigned to genera of putative fermenting bacteria, including *Amycolatopsis* and *Nocardiopsis*. These three samples all had similar moisture contents (15.8%, 13.8%, and 12.4%), and samples T1 and T7 had very similar water activities (0.86, and 0.84; Supplemental Figure 5). The remaining Tsukinuno bentonite samples, T2, T5, T8, T9, and T4, had 16S rRNA gene profiles that suggested that these samples supported primarily chemolithoautotrophic acidophilic microorganisms. The pH of sodium bentonite suspensions is typically between 9-10 (Kaufhold et al. 2008), so the detection

of DNA from acidophiles was unexpected. The FAPROTAX predictions for the 16S rRNA gene profile of sample T2 suggested a community comprised primarily of sulfur-compound oxidizers, with the rarer members capable of chemoheterotrophic metabolisms (Figure 5). The most dominant ASVs observed in multiple replicates of this sample were associated with the genera *Hydrogenophilaceae* and *Thiobacillus*. Sample T5 and T9 had 16S rRNA gene profiles dominated by an ASV classified as the acidophilic chemolithoautotroph *Acidithiobacillus ferrooxidans* (Figure 4), a species of bacteria known for its role in bioleaching in acidified mine drainages (Valdés et al. 2008), capable of the aerobic oxidation of Fe^{2+} , sulfur-compounds (Quatrini et al. 2009), hydrogen (Drobner et al. 1990), and formate (Pronk et al. 1991), and the respiration of Fe^{3+} (Sugio et al. 1985) or S^0 (Ng et al. 2000) in the absence of oxygen. This species has also been identified as a primary producer in environments where it is found due to its ability to fix nitrogen and carbon from N_2 (Mackintosh 1978) and CO_2 (Heinhorst et al. 2002), respectively. The potential for sulfur and iron oxidation by the microbial community in sample T5 was supported by FAPROTAX predictions (Figure 5). The 16S rRNA gene profiles of samples T8 and T9 included several ASVs associated with acidophilic bacteria, including *Acidiferrobacter thiooxydans*, which is obligately autotrophic and facultatively anaerobic, capable of oxidizing S^0 - or Fe^{2+} while respiring O_2 or Fe^{3+} (Hallberg et al. 2011). The 16S rRNA gene profile of sample T4 was dominated by three archaeal ASVs from the family *Thermoplasmataceae*. These ASVs are phylogenetically dissimilar to ASVs abundant in the remaining Tsukinuno bentonite clay samples. All species within the family *Thermoplasmataceae* are considered acidophiles, some having the lowest known pH optima of any species (Golyshina et al. 2016). Members of the family are facultatively anaerobic and can respire S^0 (Seegerer et al. 1988) or Fe^{3+} (Golyshina et al. 2009) in the absence of O_2 . Several species are organotrophs

(Segerer et al. 1988, Golyshina et al. 2009, 2016) whereas others gain energy from the oxidation of Fe^{2+} (Golyshina et al. 2009). The presence of sulfide oxidizing microorganisms in a DGR could promote the removal of sulfides and reduce the risk of MIC of UFCs.

Comparing to other clay deposits, a previous experiment assessing the microbial composition of the Belgian Boom clay deposit by sequencing 16S rRNA genes found the clay samples to be dominated by *Proteobacteria*, similar to samples T3, T2, T5, T8, T9, and *Firmicutes*, although several of the *Firmicutes* identified were suggested to be contaminants (Boivin-Jahns et al. 1996). Members of the phylum *Proteobacteria* were also found to be dominant members of the microbial community in Spanish bentonite clay deposits in another study, making up between 30% and 100% of total 16S rRNA gene reads (Lopez-Fernandez et al. 2015). With the exception of *Euryarchaeota* and *Tenericutes*, all phyla detected in the Tsukinuno bentonite clay samples at or above 5% abundance were also detected in the Spanish bentonite samples (Lopez-Fernandez et al. 2015). In contrast, a Chinese bentonite sample was found to have a microbial community dominated by *Thaumarchaeota* (e.g., now class *Nitrosphaeria*), specifically members of the genus *Nitrososphaera*, although *Proteobacteria* still comprise approximately 15% of the microbial community (Liu et al. 2019). All phyla detected in the Tsukinuno bentonite clay samples at or above 5% abundance, with the exception of *Tenericutes*, were also detected in the Chinese bentonite sample (Liu et al. 2019).

The only ASVs associated with sulfur reduction that were prevalent across the replicates of a sample were those classified as *Acidithiobacillus ferrooxidans*, the genus *Acidithiobacillus*, and the family *Thermoplasmataceae*, which were prevalent in samples T5, T9, and T4,

respectively. All of these bacteria and archaea are facultatively anaerobic and thus likely using O_2 instead of S^0 as an electron acceptor in the oxic locations from which the samples were taken. No ASVs associated with known SRB were detected.

Despite originating from similar locations, the Tsukinuno bentonite clay samples varied in moisture content and water activity, and microbial abundance, composition, and diversity. The physical and microbial heterogeneity of the samples highlights the importance of studying a variety of different ancient bentonite samples with biogeochemical properties spanning the range of expected properties for DGR-associated bentonite. Additionally, future studies could incorporate a chemical analysis of associated ground water, and measurements of relevant clay properties (e.g., pH, sulfur, and iron concentrations) to potentially explain microbial sample heterogeneity. Even though the taxa detected in the Tsukinuno bentonite samples by direct 16S rRNA gene sequencing are capable of some of the forms of metabolism expected to be possible in a DGR (Supplemental Figure 2), many, especially those associated with sulfate reduction, were not detected in the Tsukinuno bentonite clay 16S rRNA gene profiles. Performing these experiments on ancient natural barrier component samples from anoxic environments could yield more information on the potential for anaerobic metabolisms, such as sulfate reduction, in these types of samples. Specifically, clay cores from the Tsukinuno bentonite deposit could be analyzed to compare pristine anoxic samples to the existing tunnel wall samples.

Microbial characterization of the Tsukinuno bentonite culture DNA extracts

Although 16S rRNA gene profiles generated from the clay DNA extracts presumably represent all living microorganisms in the clay, it is important to note that not all ASVs detected

necessarily represent living or active microorganisms. Microorganisms can exist in a living but metabolically inactive state during exposure to unfavourable conditions (Zhou et al. 2019), other bacteria can form spores when conditions are not favourable (Young and Fitz-James 1959), and bacterial “relic” DNA can be preserved in the environment for thousands of years after an organism has died (Willerslev et al. 2007).

In contrast to nucleic acid-based techniques, cultivation-based approaches can test the viability of the microorganisms present in a sample, even though they are biased for microorganisms capable of growing under the given conditions. The detection of culturable microorganisms from all Tsukinuno bentonite samples demonstrates that at least a portion of the 16S rRNA genes detected in the clay was derived from living cells, either active or inactive.

Using only three cultivation approaches for a metabolically diverse community, it was expected that only a subset of the ASVs detected in the 16S rRNA gene profiles of the clay samples would be detected in the 16S rRNA gene profiles of the cultures. The occurrence of very few ASVs in the 16S rRNA gene profiles of the cultures was indeed observed, with these profiles each being dominated by one ASV representing ~70-100% of the reads (Figure 4). Several cultures had additional ASVs that were taxonomically similar to the dominant ASV. Only one ASV present in the 16S rRNA gene profile of a culture was also found in its corresponding clay 16S rRNA gene profile. This ASV, associated with the genus *Geothrix*, was found in the 16S rRNA gene profile of the anaerobic heterotroph culture of sample T2 at 0.5% abundance, and one of the 40 PCR cycle replicates of the corresponding clay sample at 0.1% abundance. Many of the microorganisms detected using 16S rRNA gene sequencing of the clay

samples were acidophilic, thermophilic, and used reduced iron and sulfur compounds as an electron source, and these conditions were not met by the cultivation techniques employed.

Sequencing of the 16S rRNA genes from culture biomass DNA extracts revealed that bacteria belonging to four different phyla (i.e., *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Firmicutes*), were isolated on the R2A agar plates incubated under oxic conditions, and additional bacteria from two phyla (i.e., *Actinobacteria* and *Firmicutes*) were isolated on R2A agar plates incubated under anoxic conditions (Figure 4). The 16S rRNA gene profile of the one positive SRB tube from sample T2 indicates that the SRB belonged to the genus *Desulfosporosinus*, which includes seven characterized species of obligately anaerobic, spore-forming SRB (Stackebrandt et al. 1997, Robertson et al. 2001, Ramamoorthy et al. 2006, Vatsurina et al. 2008, Lee et al. 2009, Alazard et al. 2010, Sánchez-Andrea et al. 2015). No ASVs assigned to the genus *Desulfosporosinus* were detected in the 16S rRNA gene profiles of the corresponding Tsukinuno bentonite clay. The oxic conditions in the mine would be expected to preclude the growth of these bacteria, although they may have existed in the location from which sample T2 was taken at a time when it was anoxic, and remained viable as spores. Members of the genus *Desulfosporosinus* have previously been identified as likely dominant sulfate reducers in clay-associated borehole fluid (Leupin et al. 2017, Engel et al. 2019b).

Both the R2A agar and the sulfate reducing medium used to culture microorganisms are complex media and would be expected to support a variety of heterotrophic metabolisms. The sulfate reducing medium is additionally enriched with sodium sulfate to support the growth of SRB. The potential for chemoheterotrophy was indeed predicted in all aerobic and anaerobic

heterotroph cultures for which a prediction could be made, with the exception of the aerobic heterotroph culture from sample T5, for which the only detected microorganism, a species of *Massilia*, was predicted to be exclusively capable of ureolysis (Figure 5). The anaerobic heterotrophs and SRB cultured from sample T2 were predicted to ferment, and the SRB culture had additional evidence for sulfur compound respiration, which could include sulfate reduction. Nitrate and nitrogen reduction potential was additionally predicted in the aerobic heterotroph culture from sample T7 (Figure 5). Sulfate reduction is of particular interest in the context of a DGR due to the production of corrosive hydrogen sulfide, and fermentation and nitrate reduction are additionally relevant as they lead to the production of gases, which could lead to fissures in the clay or rock.

Because cultivation approaches were not possible for the Opalinus Clay and NOCR samples, it is unknown whether any of the microorganisms quantified in these samples were viable or even extant. There are cultivation-independent techniques that could be applied to differentiate living cells from dead cells or relic biomarkers. For example, the adenosine triphosphate (ATP) assay, which makes use of the short-lived nature of ATP outside of a living cell, and DNA extraction modified to include a propidium monoazide incubation step, which excludes DNA not protected by an intact cell membrane from downstream analyses, have been used to study the living cells in other environments (Tian et al. 2017), and could be optimized for use in DGR-relevant environments.

Microbial characterization of NOCR and Opalinus Clay DNA extracts

The NOCR and Opalinus clay samples are both proposed host rocks for the placement of a DGR in their respective countries of origin. Five replicates of the Opalinus Clay sample had very few overlapping ASVs in the 16S rRNA gene profiles (Figure 6). The majority of ASVs detected at or above 5% abundance belonged to the phylum *Proteobacteria*, though one member each of the phyla *Tenericutes*, *Firmicutes*, and *Chloroflexi*, and two members each of the phyla *Bacteroidetes* and *Actinobacteria* were also detected. Of the 18 ASVs detected across all replicates at or above 5% abundance, only two were detected in more than one replicate (Figure 6), one of which (classified as *Vibrio*) was also detected in a corresponding negative control at a lower relative abundance. Only one other ASV (classified as *Afipia*), detected in any Opalinus Clay replicate at or above 5% relative abundance was also detected in a corresponding negative control despite the inconsistency between replicates.

For each of the three NOCR samples, the two inner layer replicates and the outer layer swab replicate had no overlapping dominant ASVs (>5% abundance; Figure 7). Most of the ASVs detected at or above 5% abundance belonged to the phylum *Firmicutes*, though some representatives of the *Proteobacteria*, *Euryarchaeota*, *Cyanobacteria*, *Bacteroidetes*, and *Actinobacteria* were also detected. Three rare ASVs were present in two replicates of NOCR sample N2, classified as *Coriobacteriales*, *Mycobacterium*, and *Pseudomonas*, respectively. None of these ASVs were detected in negative controls associated with these samples. Two ASVs detected in NOCR samples were assigned to the chloroplast of *Austrotaxus spicata*, which is a species of plant endemic to New Caledonia (a French territory in the South-Pacific) (Bobrov et al. 2004). It is not clear why DNA from this organism was found in these samples and we cannot rule out contamination. Similar to the Opalinus clay sample, only two of the ASVs (i.e.,

Pseudomonas 6533 and *Planococcaceae* 7786) were detected at or above 5% relative abundance in the NOCR samples were also detected in the corresponding negative controls, despite the low consistency between replicate 16S rRNA gene profiles. The lack of consistency between replicate 16S rRNA gene profiles of the NOCR and Opalinus Clay samples reduces confidence that detected ASVs were specifically associated with low biomass rock and clay cores and not instead contaminant sequences. A similar number of reads obtained for NOCR, Opalinus Clay and negative controls (Supplemental Figure 6) further supports a lack of sample-specific profiles, given exceedingly low rock-specific biomass. While the lack of rock-specific biomass is not unexpected given the geosphere from which samples were collected, this result is important as a benchmark for future experiments in Canada and internationally. Future work will emplace engineered barrier components *in situ*, in the NOCR and Opalinus Clay geospheres and employ similar analysis to that described above. Therefore, all future data produced in this program must be analyzed in the context of host rock formations with exceedingly low, if not absent microbial communities.

Conclusion

The potential for sulfate reduction by SRB in the bentonite clay associated with the DGR is of particular importance to DGR longevity. Both the culturing and DNA sequencing results revealed the presence of SRB in only one of the Tsukinuno bentonite samples, at very low abundance. The absence or low abundance of SRB in all natural analogue bentonite samples could imply low risk of microbiologically influenced corrosion of the copper coated UFCs. That said, the samples were taken from oxic locations and SRB are obligate anaerobes. Aerobic and anaerobic heterotrophs were detected in all samples, and there was some evidence of possible

sulfur cycling metabolisms based on the FAPROTAX predictions. The samples with the most consistent replicate 16S rRNA gene profiles were dominated by ASVs associated with chemolithoautotrophic acidophiles and heterotrophic archaeal and bacterial taxa. Although the results of this study suggest that the culturing techniques used may target the rare members of the microbial community, and that much of the potential diversity of these samples may be missed using culturing techniques alone, it is also possible that some of the additional taxa detected using 16S rRNA gene sequencing could be cultured if more relevant culturing techniques were used (e.g. acidic conditions, inorganic electron sources). The discrepancies observed between culturing and DNA-based techniques highlights the need to use additional molecular techniques, such as the ATP assay or a modified DNA extraction protocol, to more accurately quantify the viable microbial communities of clay and rock samples. To help inform DGR design considerations based on anticipated microbial communities, additional natural barrier analogue samples taken from more DGR-relevant locations (e.g. anoxic locations) should be studied to better inform the potential for the growth of key microorganisms such as SRB in a future DGR.

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Table 1. List of Tsukinuno bentonite samples and site characteristics. DNA concentrations below the detection limit (BDL) of the Qubit fluorometer with 10 μ L of sample input (8 ng/gram of clay) are indicated.

Sample	Appearance	Description	Sampling site	Clay bed	Average DNA yield (ng) per gram of clay
T1	Rock	Hand specimen taken from the bentonite exposed at the tunnel wall. Sample location 26-27cm up from the lower bentonite-shale contact.	1	19	BDL
T2	Soft	Sample taken from a very wet tunnel wall. South side of the tunnel, pH around 6-7.	1	17	104
T3	Stones	Samples taken from tunnel wall, very dry bentonite. No drilling at this site.	2	2	BDL
T4	Soft	Samples taken from cleaned tunnel wall (15 cm from original surface).	1	20	44
T5	Rock and soft	Yellow bentonite, at lower contact of bed #19.	1	19	BDL
T6	Rock and soft	Gray bentonite, 20 cm from lower contact.	1	19	BDL
T7	Rock and soft	Brownish bentonite, 50 cm from lower contact.	1	19	BDL
T8	Rock and soft	Orange bentonite, 65 cm from lower contact.	1	19	16
T9	Rock and soft	Yellow bentonite, upper contact of bed #19.	1	19	8
T10	Rock	Dry bentonite from upper contact of bed #29.	3	29	BDL

Figure 1. Abundance of 16S rRNA gene copies, culturable aerobic heterotrophs, anaerobic heterotrophs, and SRB in the Tsukinuno bentonite samples, and abundance of 16S rRNA gene copies in the NOCR and Opalinus samples. The abundance of 16S rRNA gene copies is reported as copies/gdw for the Tsukinuno samples, and as copies/g for the NOCR and Opalinus samples. Measures of CFU/g below 2500 are below the limit of confident detection of the method. The error bars represent standard deviation of technical duplicates for measures of 16S rRNA gene copies/gdw, standard deviation between calculated CFU/gdw on triplicate R2A agar plates for measures of CFU/gdw, and confidence intervals specified by the MPN table for measures of MPN/gdw. See Table 1 for additional sample information.

Figure 2. Scatterplots showing the relationship between the number of 16S rRNA gene copies/gdw and the moisture content (A) or water activity (B) of the Tsukinuno bentonite samples. The number of 16S rRNA gene copies/gdw was correlated to moisture content and water activity with Pearson correlation of 0.72 and Spearman correlation of 0.77, respectively.

Figure 3. PCoA ordination based on Bray Curtis distances of the Tsukinuno bentonite samples appended with taxa and environmental information (triplot). Genera greater than or equal to 5% relative abundance were displayed as clear circles. Environmental variables were displayed if they passed the goodness of fit statistic (R^2) threshold of 0.2. Water activity, moisture content, and DNA concentration had R^2 values of 0.2, 0.7, and 0.8 respectively. The position of the taxa bubbles represents the relative abundance-weighted average of PCoA coordinates of samples in which the taxa was present. The size of the taxa bubbles represents the percentage of reads in the

dataset corresponding to the given taxa. Samples were rarefied to 5100 reads. See Table 1 for additional sample information.

Figure 4. Bubble plot showing the 16S rRNA gene profiles of the Tsukinuno bentonite clay samples (blue) and the corresponding aerobic heterotroph (AH), anaerobic heterotroph (AnH), and SRB cultures (grey). The number in each bubble represents the relative abundance of that ASV in the particular sample. Only ASVs at or above 5% relative abundance in a sample are included. Up to six replicates were analyzed for each of the ten bentonite samples, comprising of duplicate extractions which were amplified at 40, 45, and 50 cycles. Samples that showed no visible amplicon in an agarose gel after PCR were not included in the sequencing analysis and thus are not included in this plot. Bed number indicates the origin of samples from clay deposits. Sample order in the figure reflects the sampling locations in the mine; adjacent samples in the figure were taken from adjacent locations in the mine. See Table 1 for additional sample information.

Figure 5. Bubble plot showing the relative abundance of ASVs predicted to have the listed functions in the Tsukinuno bentonite samples and the corresponding aerobic heterotroph (AH), anaerobic heterotroph (AnH) and SRB cultures. The number in each bubble represents the relative abundance of that predicted function in the particular sample. Functions that occurred at less than 1% relative abundance in a sample were not included. Predicted functions were made by FAPROTAX (Louca et al. 2016) and were based on the sequenced V4-V5 region of the 16S rRNA gene. Bed number indicates the origin of samples from clay deposits. See Table 1 for additional sample information.

Figure 6. Bubble plot showing the 16S rRNA gene profile of the Opalinus clay sample. The labels 40, 45, and 50 indicate the number of PCR cycles used to amplify the DNA samples. The number in each bubble represents the relative abundance of that ASV in the particular sample. Only ASVs at or above 5% relative abundance in a sample are included. Replicates that showed no visible amplicon in an agarose gel after PCR were not included in the sequencing analysis and thus are not included in this plot.

Figure 7. Bubble plot showing the 16S rRNA gene profile of the inner and swabbed outer layer of the three Northern Ontario crystalline rock (NOCR) samples. Two biological replicates of each rock sample are included. The number in each bubble represents the relative abundance of that ASV in the particular sample. Only ASVs at or above 5% relative abundance in a sample are included.

ASV taxonomic affiliation

Predicted function

Phylum

- Unclassified
- Actinobacteria
- Bacteroidetes
- Chloroflexi
- Firmicutes
- Proteobacteria
- Tenericutes

Biological replicate

- 1
- 2