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# Microbial diversity gradients in the geothermal mud volcano underlying the hypersaline Urania Basin

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Mud volcanoes transport deep fluidized sediment and their microbial communities and thus provide a window into the deep biosphere. However, mud volcanoes are commonly sampled at the surface and not probed at greater depths, with the consequence that their internal geochemistry and microbiology remain hidden from view. Urania Basin, a hypersaline seafloor basin in the Mediterranean, harbors a mud volcano that erupts fluidized mud into the brine. The vertical mud pipe was amenable to shipboard Niskin bottle and multicorer sampling and provided an opportunity to investigate the downward sequence of bacterial and archaeal communities of the Urania Basin brine, fluid mud layers and consolidated subsurface sediments using 16S rRNA gene sequencing. These microbial communities show characteristic, habitat-related trends as they change throughout the sample series, from extremely halophilic bacteria (KB1) and archaea (*Halodesulfoarchaeum* spp.) in the brine, toward moderately halophilic and thermophilic endospore-forming bacteria and uncultured archaeal lineages in the mud fluid, and finally ending in aromatics-oxidizing bacteria, uncultured spore formers, and heterotrophic subsurface archaea (Thermoplasmatales, Bathyarchaeota, and Lokiarchaeota) in the deep subsurface sediment at the bottom of the mud volcano. Since these bacterial and archaeal lineages are mostly anaerobic heterotrophic fermenters, the microbial ecosystem in the brine and fluidized mud functions as a layered fermenter for the degradation of sedimentary biomass and hydrocarbons. By spreading spore-forming, thermophilic Firmicutes during eruptions, the Urania Basin mud volcano likely functions as a source of endospores that occur widely in cold seafloor sediments.

## KEYWORDS

archaea, bacteria, hypersaline basin, cold seep, marine subsurface sediments, Urania Basin

# 1 Introduction

The Urania Basin is situated southwest off the coast of Crete, in the inner plateau of the Mediterranean Ridge characterized by the presence of Messinian salt evaporites within the sediments, hundreds of meters below the seafloor (Westbrook and Reston, 2002). With a chloride content of 120 g/L, i.e., more than five times of that in Mediterranean seawater, Urania Basin brine is the least saline of the Mediterranean DHABs (deep hypersaline anoxic basin); yet Urania Basin brine contains exceptionally high levels of methane (up to 5.56 mM) and sulfide (up to 16 mM) (Borin et al., 2009), making Urania Basin one of the most sulfidic marine water bodies on Earth (Karisiddaiah, 2000; Charlou et al., 2003). Despite these extreme conditions, active microbial communities are present (van der Wielen et al., 2005) and contribute to biogeochemical cycling of carbon and sulfur in the deep anoxic hypersaline brine of the Urania Basin.

A unique feature of the Urania Basin is the presence of an active mud volcano in the southwestern part of the horseshoe-shaped basin, found by a CTD survey (conductivity, temperature, depth) during the R/V Urania expedition that discovered the basin in 1993. Elevated temperatures of up to 55°C coincide with active mud volcanism underneath the brine layer (Corselli et al., 1996; Fusi et al., 1996). A direct association between brines and active mud volcanism has been previously documented by exotic deposits sampled in the outer rim of the basin (Hübner et al., 2003) and by the presence of sediments containing Plio-Pleistocene microfossils at the bottom of the basin (Aloisi et al., 2006). The fluidized mud volcano sediment, consisting of micrometer-sized particles, nanofossils, carbonate and gypsum grains, constitutes a convectively mixed suspended seafloor that extends vertically over 110 m before transitioning into solidified subsurface sediment (Aiello et al., 2020).

The Urania Basin mud volcano provides a valuable model system to test the working hypothesis that mud volcanoes bring deep subsurface microorganisms to the surface and distribute them across the deep-sea floor. As conduits of fluid migration from the deep subsurface to the seafloor, mud volcanoes provide windows into the deep subseafloor biosphere (Hoshino et al., 2017) that is otherwise only accessible by deep drilling. Since the chemistry of the discharging fluidized muds varies within and between sites, different combinations of brine, methane, gaseous and liquid hydrocarbons, sulfides, nutrients and low-molecular weight organic acids shape the resident microbial communities and their activities, as shown in numerous individual studies (Joye et al., 2009; Pachiadaki et al., 2010, 2011; Lazar et al., 2011a,b, 2012; Pachiadaki and Kormas, 2013). Mud volcanoes are pulsating with fluidized mud that frequently overflows their surrounding seafloor basins (McDonald et al., 2000; Hübner et al., 2003; Teske and Joye, 2020) and by the same token deposit fluid mud-hosted bacteria and archaea. Mud volcano and cold seep geofluids have been proposed as the carrier medium for seeding surficial seafloor sediments with endospore-forming

thermophilic Firmicutes that could not grow under cold *in situ* conditions (Hubert et al., 2009; Rattray et al., 2022).

To explore the spatially shifting microbial community in the Urania Basin brine/mud volcano system, we analyzed bacterial and archaeal 16S rRNA gene amplicons along a vertical profile from consolidated subseafloor sediments at the underlying seabed at the bottom of the mud volcano (the “pit”), through fluidized mud volcano sediment layers that extend from the subsurface seabed to the bottom of the brine lake, to the overlying brine lake. This approach was supported by analysis of gaseous hydrocarbons and hydrocarbon lipid biomarkers from selected sediment samples. A previous 16S rRNA gene sequencing survey of deep mud fluid sediment has uncovered diverse bacterial and archaeal populations (Yakimov et al., 2007) but did not produce a microbial profile through all three layers (subsurface sediment, fluid mud layers, brine fluid). The geochemical and microbiological changes observed along this depth profile indicate that each sediment and brine layer has selected for its unique microbial community.

## 2 Materials and methods

### 2.1 Sediment sampling

Brine, mud fluids and subseafloor sediments were sampled in the Urania Basin during cruise M84/1 (DARCSEAS) of R/V Meteor in February 2011. Two CTD casts GeoB15101-1 and GeoB15101-6 (35°13.86'N, 21°28.30'E) deployed twenty-four Niskin bottles each at increasing water depths, and samples were used for stable ion measurements (Aiello et al., 2020) and DNA extractions, respectively. Based on stable ion concentrations and CTD pressure readings, the seawater-brine interface was located between 3,461 and 3,463 m water depth (mwd), and the brine/fluid mud interface between 3,607 and 3,610 m water depth (Aiello et al., 2020). Within the fluid mud layers, sampling depths were conservatively based on CTD cable length since the high density of the mud interfered with reliable pressure measurements by the CTD sensors as soon as the CTD entered the fluid mud layer. For GeoB15101-1, bottles 1 (70 m water depth, mwd) to 8 (3,461 mwd) sampled the overlying seawater, whereas bottles 9 (3,463 mwd) to 24 (3,598 mwd) sampled the hypersaline brine lake. For cast GeoB15101-6 (used in this study), bottle 1 sampled overlying seawater (3,000 mwd), and bottles 2 (3,560 mwd) to 7 (3,605 mwd) sampled the hypersaline brine. The underlying mud fluid layers were sampled in 10-m intervals by bottles 8 (3,610 m cable length) to 20 (3,730 m cable length). The deepest mud fluid sample, no. 21, was collected at a cable length of 3,736 m when the sediment became sufficiently consolidated to prevent further sinking of the CTD. Subsamples taken from the 24 Niskin bottles of the CTD cast GeoB15101-6—sampled progressively from the seawater, through the Urania brine lake, down to the seafloor—clearly demonstrated the fluid mud layer starting with bottle 8, and

continuing downcore toward gradually darker mud (Figure 1). The bottom of the fluidized mud layer (sample 21, 3,736 m cable length) was 110 m below the expected seafloor depth, where brine transitions into mud, based on echo-sounder data (Zabel, 2012).

The subsequently deployed gravity core GeoB15101-7 (35°13.87'N, 21°28.30'E) recovered 4.5 m of the consolidated sediments below the mud fluid layers, and depths for these sediment samples are given in cm from the top of the core. Subsamples of each Niskin bottle of cast GeoB15101-6 as well as selected gravity core sections of cast GeoB15101-7 were immediately stored at -20°C for subsequent molecular analyses, including hydrocarbon lipid biomarkers. Concentration measurements of methane and ethane were carried out onboard the ship, whereas investigation of the gaseous hydrocarbon's stable carbon isotopes was performed in the home lab.

## 2.2 Hydrocarbon measurements

Concentrations of dissolved methane and ethane were determined according to previously reported protocols (Pimmel and Claypool, 2001; D'Hondt et al., 2003), using two to three mL of brine, mud fluid and subseafloor sediment samples (see Supplementary Methods for details). Isotope values of methane and ethane are reported in the delta notation ( $\delta^{13}\text{C}$ ) relative to the Vienna Pee Dee Belemnite (VPDB) standard using a calibrated in-house  $\text{CO}_2$  reference gas standard. For hydrocarbon lipid biomarker analysis of two gravity core sediments samples from 10 to 30 and 260–280 cm core depth, 50 g of freeze-dried sample material was extracted using a modified Bligh and Dyer protocol (Sturt et al., 2004). Hydrocarbons were identified *via* their known mass spectra and concentrations were calculated from their peak area in the FID chromatogram relative to the injection standard (see Supplementary Methods for details).

## 2.3 DNA extraction, PCR amplification and 16S rRNA gene library construction

Samples chosen for molecular analyses included Niskin bottles 5 (3,590 mwd, hypersaline brine), 8 (3,610 m, top of the mud fluid layer), 14 (3,670 m, middle of the mud fluid layer) and 21 (3,736 m, bottom of the mud fluid layer) from the GeoB15101-6 CTD cast (Figure 1), and depth sections 10–30 and 260–280 cmbsf of the GeoB15101-7 gravity core. DNA was extracted using a bead beating soil extraction kit on 10 g of wet sediment for the mud fluid and gravity core samples (UltraClean<sup>®</sup> Mega Soil DNA Isolation Kit, MO BIO, CA now QIAGEN), and using 50 mL of filtered brine (PowerSoil<sup>®</sup> DNA Isolation Kit, Mo BIO, CA now QIAGEN). The extracted DNA

was then purified using the Wizard DNA Clean-Up System (Promega, Wisconsin, USA).

Archaeal 16S rRNA genes were amplified using the primers A24F (5'-CGGTTGATCCTGCCGGA-3') and A1492R (5'-GGCTACCTTGTTACGACTT-3') (Casamayor et al., 2000) and the Flexi GoTaq DNA polymerase (Promega, WI). A total of 35 PCR cycles (1 min denaturation at 94°C, 1'30 min annealing at 50°C, and 2 min elongation at 72°C) were run in a BIO-RAD iCycler (Hercules, CA). A nested PCR using the primer set A24F (5'-CGGTTGATCCTGCCGGA-3'; Casamayor et al., 2000) and Arch915R (5'-GTGCTCCCCCGCCAATTCCT-3', Stahl and Amann, 1991) was necessary in order to obtain sufficient amounts of archaeal 16S rRNA gene amplicons for cloning, using the following conditions: 1 min denaturation at 94°C, 1'30 min annealing at 58°C, and 2 min elongation at 72°C, repeated for 35 cycles.

Bacterial 16S rRNA genes were amplified using the B8F-B1492R primer set (5'-AGRGTTTGATCCTGGCTCAG-3 and 5'-CGGCTACCTTGTTACGACTT-3; Teske et al., 2002) using the following PCR conditions: 1 min denaturation at 94°C, 1'30 min annealing at 50°C, and 2 min elongation at 72°C, repeated for 26 cycles to limit contamination since the extracted DNA concentrations were extremely low. A nested PCR using the primer combination B27F (5'-AGAGTTTGATCCTGGCTCAG-3') and U1406R (5'-GACGGCGGTGTGTRCA-3'; Heuer et al., 1997) was necessary to obtain sufficient amplicon material for cloning, using the following conditions: 1 min denaturation at 94°C, 1'30 min annealing at 50°C, and 2 min elongation at 72°C, repeated for 30 cycles. PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, CA, USA) prior to cloning, and were then cloned using the TOPO XL cloning kit (Invitrogen, CA). After inserting the PCR amplified genes into the provided plasmid, the plasmid DNA was incorporated into *Escherichia coli* TOPO10 One Shot cells. Transformed cells were grown overnight, and colonies were collected for Sanger sequencing.

## 2.4 DNA sequencing and phylogenetic analysis

The microbial census of the Urania Basin mud layers is deliberately based on nearly full-length 16S rRNA gene clone libraries for Bacteria (1,379 bp in *E. coli* standard notation) and widely used large fragments for archaea (890 bp for the Archaea), in order not to compromise the phylogenetic placement and taxonomic identification of potentially novel microbial populations in this unusual extreme habitat, and to provide a well-curated reference dataset for 16S-rRNA based comparison that is not limited by sequence length. Furthermore, given the nature of the extremely hypersaline samples, DNA analyses *via* high-throughput sequencing revealed themselves to be ineffective by only yielding a dozen reads.

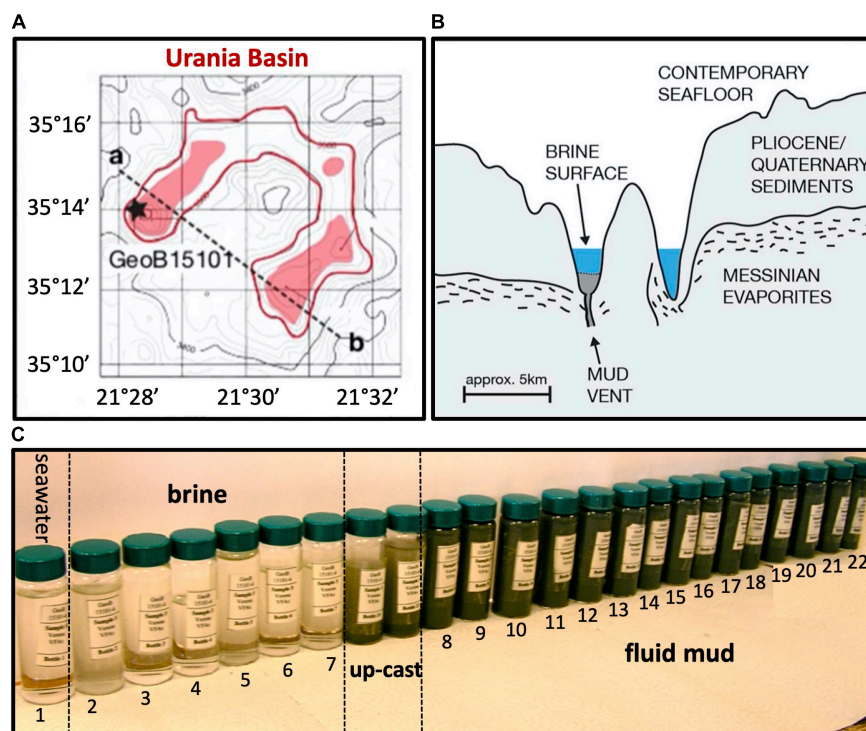


FIGURE 1

(A) Lower left, bathymetry of Urania Basin with bottom brines highlighted in red, and profile line drawn from the mud volcano in the northwestern end toward the southeastern end of Urania Basin. (B) Geologic sketch of Urania Basin, following the profile line between points a and b. (C) Sample vials with Niskin bottle samples from seawater, brine, and fluidized mud. The samples between brine and mud layer represent perturbed samples from the CTD upcast (GeoB15101-6, Zabel, 2012) at 3,605 and 3,610 m depth. All figure panels modified from Aiello et al. (2020).

Sequences were obtained by Genewiz (South Plainfield, NJ) on an ABI Prism 3730xl sequencer using the M13R primer. Chimeras were identified with Mothur's chimera slayer feature (Schloss et al., 2009), complemented by Blast analysis of sequence segments, and removed from phylogeny inferences. For initial phylogenetic analyses, the 16S rRNA gene sequences were edited in the BioEdit v7.0.5 program (Hall, 1999) and aligned by using the SINA webaligner (Pruesse et al., 2012).<sup>1</sup> The alignment was then manually checked using ARB (Ludwig et al., 2004) and initial phylogenetic trees were calculated using the neighbor-joining method in ARB. Robustness of the inferred topology was tested by bootstrap resampling with 1,000 replicates. Final phylogeny figures were constructed with sequences selected from cultured bacteria and archaea and complemented by environmental sequences (limited to ca. 50–80 sequences for readability), and manually aligned for secondary rRNA structure to ensure consistent comparisons of homologous nucleotide positions across variable and hypervariable regions (Schloss, 2013); subsequently, tree topology was checked by 1,000 bootstrap

replicates (Swofford, 2000). Whenever possible, pure-culture isolates with close sequence similarity to Urania Basin clones (for genus level > 95%, Schloss and Handelsman, 2005) were preferred for phylogenetic comparisons, to limit the inherent uncertainties of sequence-based physiological inferences (see Langille et al., 2013 for discussion). For uncultured lineages, Urania Basin sequences were identified as specifically as possible by comparisons to published subgroup analyses and phylogenies, with metagenomes when possible. The sequence data reported here were submitted to the GenBank nucleotide sequence database under the accession numbers OP352539-OP352606, OP352609-OP352677, OP352609-OP352677, OP352680-OP352749, OP352780-OP352844, OP389299-OP389989, and OP856603-OP856629.

### 3 Results

#### 3.1 Depth stratification and biogeochemistry

During CTD deployment, a prominent halocline was detected at about 3,465 mwd, and corresponded to a salinity

<sup>1</sup> <http://www.arb-silva.de/aligner>

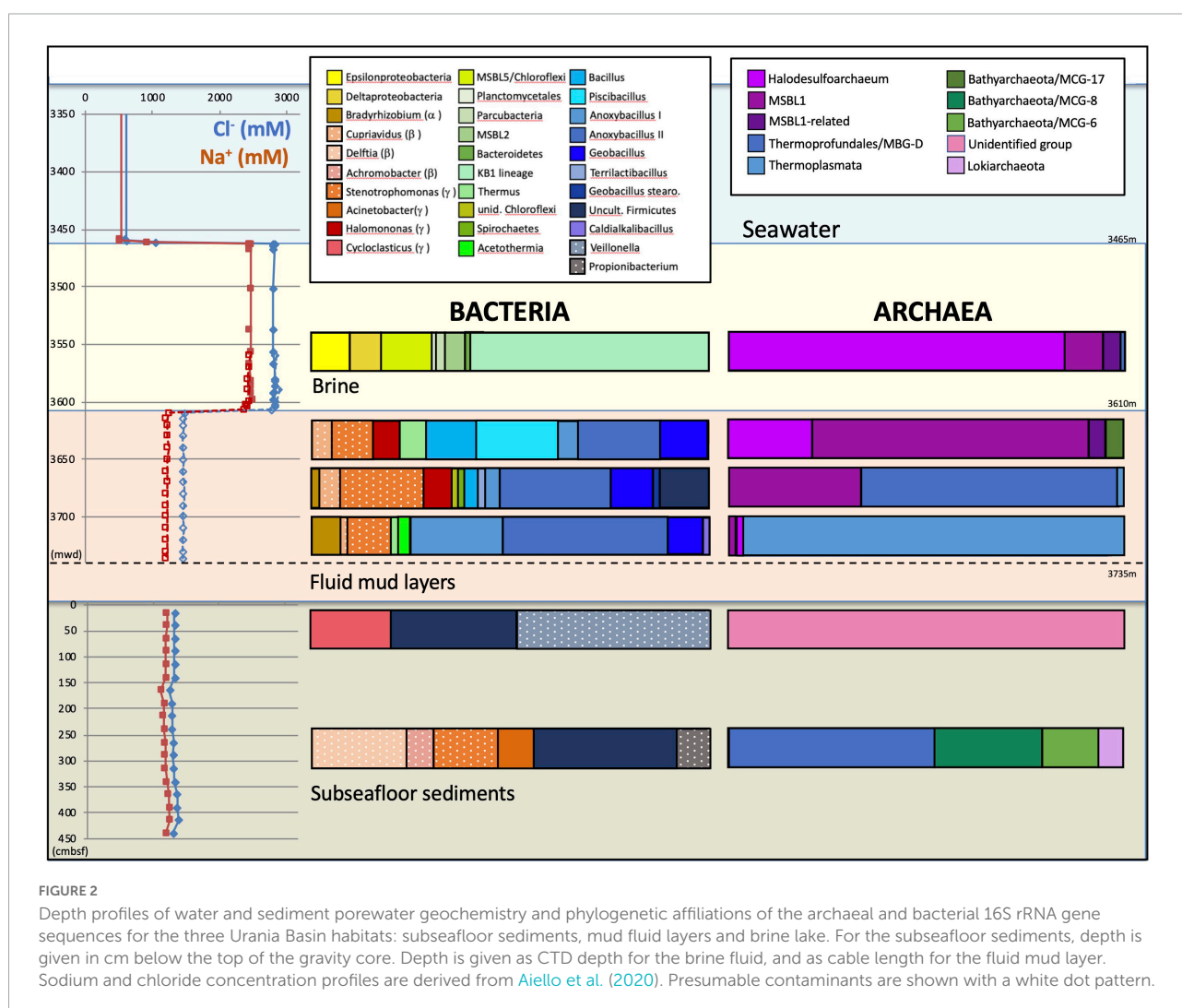


increase from ~39 to ~152 PSU (practical salinity unit), a 2.5°C increase in temperature (14–16.5°C) and the complete depletion of free oxygen; this transition marks the seawater-brine interface (Zabel, 2012). Once the CTD cast went through the brine layer and reached the depth of the seafloor, identified by onboard echo sounder at approximately 3,600 m, the cable tension did not drop, and the deployment was continued below that depth. About 160 m within the brine, at 3,625 mwd, a second density boundary was detected below which the CTD malfunctioned, and from then on depth was measured in cable length. The deployment continued until 3,735 m cable length, when a relatively fast decrease of the cable tension indicated a third boundary as the CTD rosette reached sufficiently consolidated sediments that stopped further sinking. This depth was 110 m below the expected seafloor depth based on echo sounder data (Zabel, 2012).

Overlying the subseafloor sediments and fluidized muds of the Urania Basin like a lid, a massive brine layer extends between 3,463 and 3,610 mwd. The samples recovered in Niskin bottles

returned clear brine fluids from the 3,465 m to 3,610 mwd interval (Figure 1). The redox potential in the brine lake was extremely negative at 3,560 mwd, i.e., -427 mV, consistent with previous results showing that oxygen was completely depleted in the reducing brine lake environment (Borin et al., 2009). The brine contained chloride and sodium at constant concentrations of 2,823 and 2,450 mM, respectively (Aiello et al., 2020), ca. 4.5 times higher than in the underlying fluid mud and subseafloor sediments (Figure 2).

For over 110 vertical meters below the brine layer, the Niskin bottles collected fluid mud (Figure 1) with much higher density (>1.6 g/cm<sup>3</sup>) than the brine (1.13 g/cm<sup>3</sup>), comparable to the bulk-saturated density of similar pelagic sediments (Fusi et al., 1996). The water content in the fluid mud ranges between ~61 and 68%, which is above the Attemberg's liquid limit measured for similar sediments recovered during ODP Leg 160 at the Olimpi Mud Volcano field (Kopf et al., 1998). The flat pore water profiles of Na<sup>+</sup> and Cl<sup>-</sup> across the fluid mud layer suggest some internal mixing



to obscure internal biogeochemical stratification (Figure 2). Internal mixing is also supported by consistent total sulfur concentrations, dominated by sulfate, near 95 and 53 mM for brine and fluid mud, respectively (Aiello et al., 2020). Above-seawater salinities suggest that the fluid mud was mixed with pre-existing brine fluids. In lakes, temperature and salt differences between two bodies of water/sediment can lead to double-diffusive convection cells, which can highly enhance vertical transport of heat and salt (Bohrer, 2012). Consistent with the maximum depth reached by CTD rosette deployment in the fluid mud layers, the Gravity corer (GeoB15101-7) encountered a resistant sediment layer at about 3,735 m cable length. The Gravity corer returned 4.5 m of sediment, soupy and homogeneous within the upper 80 cm but increasingly firm below; the sediment at the very bottom of the core was consolidated.

### 3.2 Gases and hydrocarbon lipid biomarkers

Previously measured temperatures of 45°C in the bottom of the southwestern part of the basin (Corselli et al., 1996) are a strong indicator of a deep subsurface reservoir fueling fluids and mud to the Urania Basin seafloor. Since high pressure and temperature transform buried organic matter to hydrocarbons, the consolidated subseafloor sediment samples obtained by gravity coring were examined for methane and ethane, as well as hydrocarbon lipid biomarkers. The concentrations of methane and ethane are consistently high, 0.14–0.79 mM and 0.03–0.13 mM in the brine, respectively, and 0.4–3.3 mM and 0.07–0.60 mM, respectively, in the consolidated subseafloor sediments (Supplementary Table 1). The  $\delta^{13}\text{C}$  values of methane and ethane in the fluid mud layers are rather invariable (approximately  $-30.7$  and  $-30.2\%$ , respectively) and nearly identical to  $\delta^{13}\text{C}$  values in the subseafloor sediment (approximately  $-30.3$  and  $\sim -29.1\%$ , respectively). Both are indicating a deep thermogenic source, which is corroborated by exceptionally low  $\text{C}_1/\text{C}_2$  ratios of  $\sim 5$  in the fluid mud and in the sediments (Supplementary Table 1).

Polycyclic aromatic molecules in subseafloor sediments (Supplementary Table 2) included compounds typically found in petroleum such as naphthalene, fluorine, phenanthrene or pyrene but also high amounts of benzidine. The portion of polyaromatic hydrocarbons was high compared to the relative sum of identified hydrocarbons, including odd n-alkanes in the range from  $\text{C}_{21}$  to  $\text{C}_{33}$  as well as terpenoids such as squalene, Hop-17,21-ene and lycopane, i.e., 82.6% in the deep subseafloor sample (260–280 cm core depth) and 86.7% in the shallow sample (10–30 cm core depth). The extremely high proportion of thermally altered hydrocarbons detected in the subseafloor sediments of the Urania Basin is consistent with the hypothesis of a deep, hot reservoir that charges deep sediments

and fluidized mud with gas of thermocatalytic or hydrothermal origin (Karisiddaiah, 2000; Hübner et al., 2003).

### 3.3 Microbial community of the Urania Basin brine

The bacterial and archaeal community of Urania basin, as evaluated by 16S rRNA gene sequencing (Table 1 and Supplementary Table 3), underwent pronounced compositional changes between brine, fluid mud and bottom sediment samples (Figure 2). Since these samples differ mainly by salinity, and less by biomarker content (only the bottom sediment) or by gas concentration and isotopic signature, microbial community differences are likely to be connected to salinity; extreme halophiles would be expected in the brine layer and might diminish in the fluid muds below.

Indeed, the uncultured halophilic Kebrut deep group 1 (KB-1) dominated the bacterial 16S rRNA data set from the brine sample (Figure 3 and Table 1). This bacterial lineage was first detected in brine sediments from the Kebrut Deep, Red Sea (Eder et al., 1999), and has so far been exclusively found in saline and hypersaline habitats, anoxic brines and sediments (Eder et al., 2001, 2002; Antunes et al., 2011; Ferrer et al., 2012). Yakimov et al. (2013) suggested that KB-1 could carry out reductive cleavage of glycine betaine and consequently produce acetate and methylamines, which could be used by halophilic methylotrophic  $\text{H}_2$ -producing methanogens, such as the putatively methanogenic uncultured MSBL-1 group (Nigro et al., 2016). KB-1 also shares the carbon monoxide dehydrogenase (subunit *CooS*) and the Acetyl-CoA synthase (subunit *AcsB*) of the  $\text{CO}_2$ -fixing acetyl-CoA pathway with *Candidatus Acetothermum autotrophicum*, a member of its sister lineage *Acetothermia* (Nigro et al., 2016), suggesting shared physiological flexibility with both autotrophic and acetogenic potential (Youssef et al., 2019). Six sequences were affiliated with sulfate-reducing bacteria (Figure 4). Three of these sequences were affiliated with the *Desulfobacterium anilini* group within the *Desulfobacteraceae*, a cluster of obligately aromatics-degrading sulfate-reducing bacteria that oxidize and remineralize substituted aromatics and polyaromatic substrates (Teske, 2019). Two sequences were related to the haloalkaliphilic genus *Desulfonatrobacter* (Sorokin et al., 2012), and one clone belonged to the family *Desulfobulbaceae*, a diverse group of incompletely oxidizing sulfate-reducing or elemental sulfur-disproportionating bacteria (Kuever et al., 2005). Generally, sulfate-reducing bacteria have access to high sulfate concentrations in the brine and fluid mud layers (Aiello et al., 2020). Eight sequences were affiliated with  $\epsilon$ -Proteobacteria, chemolithoautotrophs that probably use the reverse TCA cycle to fix carbon, and usually thrive in sulfidic interface environments, for example the redoxcline of sulfidic waters (Schmidtova et al., 2009). Four sequences

**TABLE 1** Summary of Urania Basin 16S rRNA gene sequences and their phylogenetic affiliations based on Genbank searches for Bacteria and Archaea.

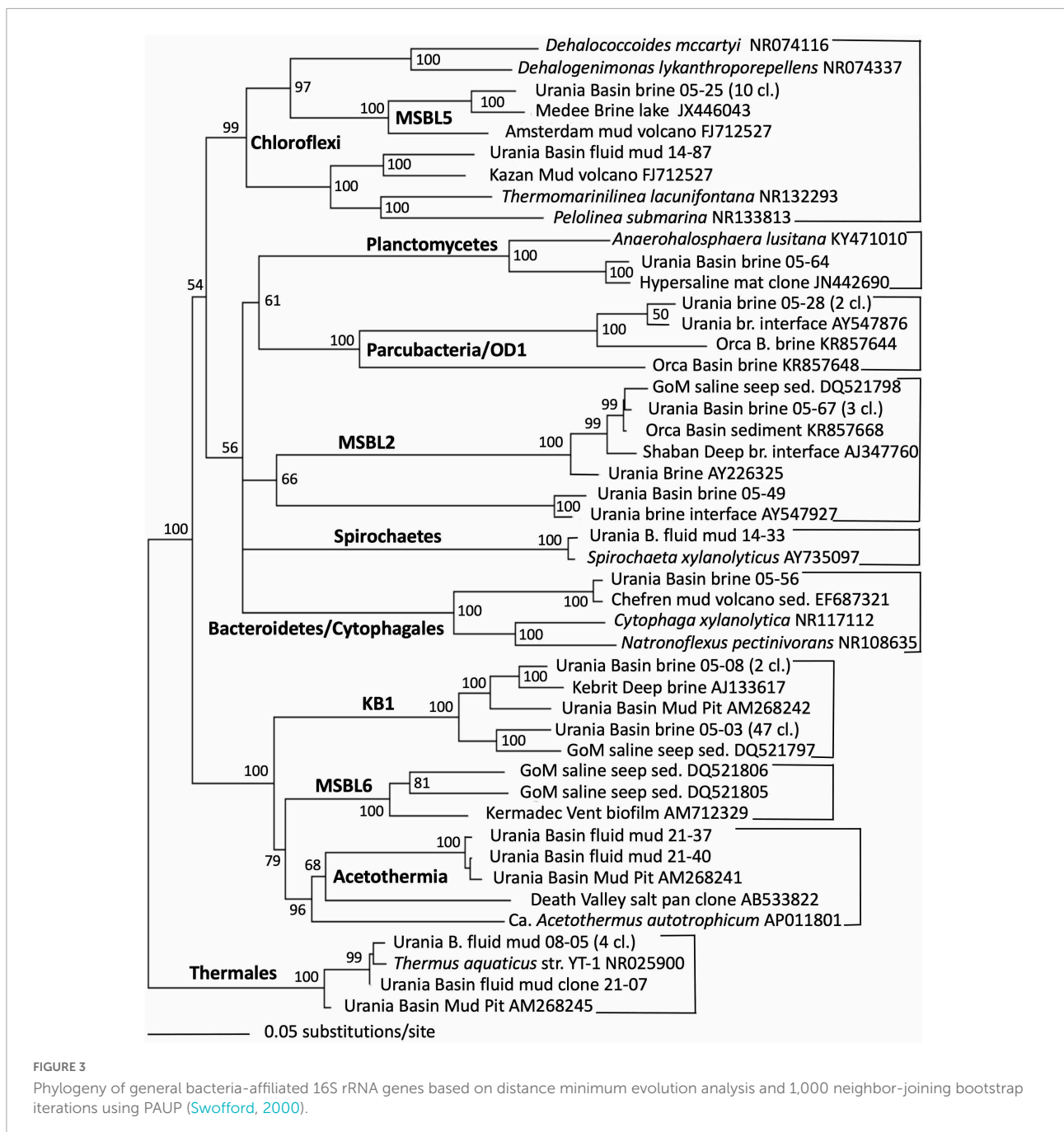
| Sample                            | Total clones                       | Proteobacteria   | Other bacteria  | Grampositives   | Archaea  |
|-----------------------------------|------------------------------------|--|---|---|--|
| Brine layer/Sample 5              | 82 bact. clones<br>93 arch. clones | 4 <i>Desulfatiglandales</i> ( $\delta$ )<br>1 <i>D. natronobacter</i> ( $\delta$ )<br>1 <i>Desulfococcus</i> sp. ( $\delta$ )<br>1 <i>Desulfobulbales</i> ( $\delta$ )<br>2 <i>Sulfurimonas</i> sp. ( $\epsilon$ )<br>2 <i>Sulfurovum</i> sp. ( $\epsilon$ )<br>4 <i>Arcobacter</i> sp. ( $\epsilon$ ) | 10 MSBL5<br>1 Planctomycetales<br>2 Parcubacteria<br>3 MSBL2<br>1 MSBL2-related<br>1 Bacteroidetes<br>2 KB1 (type 1)<br>47 KB1 (type 2) |   | 79 <i>Halodesulfoarchaeum</i> sp.<br>9 MSBL1<br>4 MSBL1 sister lineage<br>1 Thermopfundales/MBGD       |
| Upper fluid mud layer/Sample 8    | 59 bact. clones<br>90 arch. clones | 6 <i>Stenotrophomonas</i> sp. ( $\gamma$ )*<br>3 <i>Cupriavidus</i> sp. ( $\beta$ )*<br>4 <i>Halomonas</i> sp. ( $\gamma$ )  | 4 <i>Thermus</i> sp.  | 7 <i>Bacillus</i> sp.<br>12 <i>Piscibacillus</i> sp.<br>3 <i>Anoxybacillus</i> sp. (near <i>A. flavithermus</i> )<br>12 <i>Anoxybacillus</i> sp. (near <i>A. geothermalis</i> )<br>7 <i>Geobacillus</i> sp. (near <i>G. pallidus</i> )  | 63 MSBL1<br>4 MSBL1 sister lineage<br>19 <i>Halodesulfoarchaeum</i> sp.<br>4 Bathyarchaeota/<br>MCG-17 |
| Central fluid mud layer/Sample 14 | 57 bact. clones<br>95 arch. clones | 1 <i>Bradyrhizobium</i> ( $\alpha$ )<br>12 <i>Stenotrophomonas</i> sp. ( $\gamma$ )*<br>3 <i>Cupriavidus</i> sp. ( $\beta$ )*<br>4 <i>Halomonas</i> sp. ( $\gamma$ )   | 1 Chloroflexi<br>1 Spirochete   | 2 <i>Bacillus</i> sp.<br>1 <i>Terrilactibacillus</i> sp.<br>2 <i>Anoxybacillus</i> sp. (near <i>A. flavithermus</i> )<br>16 <i>Anoxybacillus</i> sp. (near <i>A. geothermalis</i> )<br>6 <i>Geobacillus</i> sp. (near <i>G. pallidus</i> )<br>1 <i>Geobacillus</i> sp. (near <i>G. stearothermophilus</i> )<br>7 uncult. Firmicutes | 32 MSBL1<br>62 Thermopfundales/MBGD<br>1 Marine Thermoplasmata   |
| Lower fluid mud layer/Sample 21   | 56 bact. clones<br>93 arch. clones | 4 <i>Bradyrhizobium</i> sp. ( $\alpha$ )<br>6 <i>Stenotrophomonas</i> sp. ( $\gamma$ )*<br>1 <i>Cupriavidus</i> sp. ( $\beta$ )*   | 2 Acetothermia<br>1 <i>Thermus</i> sp.  | 12 <i>Anoxybacillus</i> sp. (near <i>A. flavithermus</i> )<br>24 <i>Anoxybacillus</i> sp. (near <i>A. geothermalis</i> )<br>5 <i>Geobacillus</i> sp. (near <i>G. pallidus</i> )<br>1 <i>Caldialkalibacillus</i> sp.   | 2 MSBL1<br>1 <i>Halodesulfoarchaeum</i> sp.<br>90 Marine Thermoplasmata                                |
| Sediment core 10–30 cm            | 80 bact. clones<br>85 arch. clones | 16 <i>Cycloclasticus</i> sp. ( $\gamma$ )  |   | 25 uncultured Firmicutes<br>39 <i>Veillonellaceae</i> sp.*  | 85 Urania Basin Euryarchaeotal Group (UBEG)  |
| Sediment core 260–280 cm          | 75 bact. clones<br>73 arch. clones | 12 <i>Stenotrophomonas</i> sp. ( $\gamma$ )*<br>18 <i>Delftia</i> sp. ( $\beta$ )*<br>7 <i>Acinetobacter</i> sp. ( $\gamma$ )<br>5 <i>Achromobacter</i> sp. ( $\beta$ )*   |   | 27 uncultured Firmicutes<br>6 <i>Propionibacterium</i> sp.*   | 38 Thermopfundales/MBGD<br>20 Bathyarchaeota/MCG-8<br>10 Bathyarchaeota/<br>MCG-6<br>5 Lokiarchaeota   |

Sequence similarities in % to Genbank matches are listed in [Supplementary Table 3](#). Asterisks identify likely contaminants.

were affiliated to the genus *Arcobacter*, efficient CO<sub>2</sub> fixers in hypoxic sulfide-rich habitats that oxidize sulfide to produce elemental sulfur while surviving under severe electron acceptor limitation (Wirsen et al., 2002). The remaining sequences were affiliated with the sulfide-oxidizing, nitrate-, or sulfur-reducing genera *Sulfurimonas* and *Sulfurovum* (Campbell et al., 2006). Sequences affiliated with uncultured, presumably halophilic bacterial lineages from seafloor brine lakes (Bannock Brine Basin) were recovered in the Urania brine, and include the MSBL5 lineage within the Chloroflexi, and the MSBL2 lineage that is currently not closely affiliated with other bacteria

(Daffonchio et al., 2006). Other bacterial community members were represented by small numbers of clones of most likely heterotrophic and/or fermentative groups among the Planctomycetes, Parcubacteria (Castelle et al., 2017), and Bacteroidetes (Figure 3 and Table 1).

The archaeal sequences in the brine were dominated by anaerobic halophiles (Figure 5 and Table 1). Archaeal 16S rRNA gene sequences in the Urania Basin brine were closely related to *Halodesulfoarchaeum formicum*, a lithoheterotroph that oxidizes formate or H<sub>2</sub> with elemental sulfur, thiosulfate or DMSO as the electron acceptor, and to *Haloanaeroarchaeum*



*sulfureducans*, which oxidizes acetate by sulfur respiration (Sorokin et al., 2018). The Urania Basin haloarchaeal counterparts to these cultured strict anaerobes are frequently recovered from the highly reducing and sulfide-rich Urania Basin brine. Other members of the archaeal brine community include members of the uncultured, presumably methanogenic MSBL1 lineage (Daffonchio et al., 2006) that grow potentially in syntrophy with KB1 bacteria (Yakimov et al., 2013), four clones of a MSBL1-related sister lineage, and a single clone of the heterotrophic Thermoprofundales (Zhou et al., 2019).

### 3.4 Microbial community of the Urania Basin fluid mud layers

The taxonomic affiliation of bacterial sequences in the fluid mud layers changed considerably from those in the brine layer, to such a degree that the clone libraries showed no overlap at all (Figure 2 and Table 1). Instead of being affiliated with diverse sulfur-cycling delta- and epsilonproteobacteria and phylum-level halophilic lineages, most bacterial sequences of the three fluid mud layers comprised sequences specifically



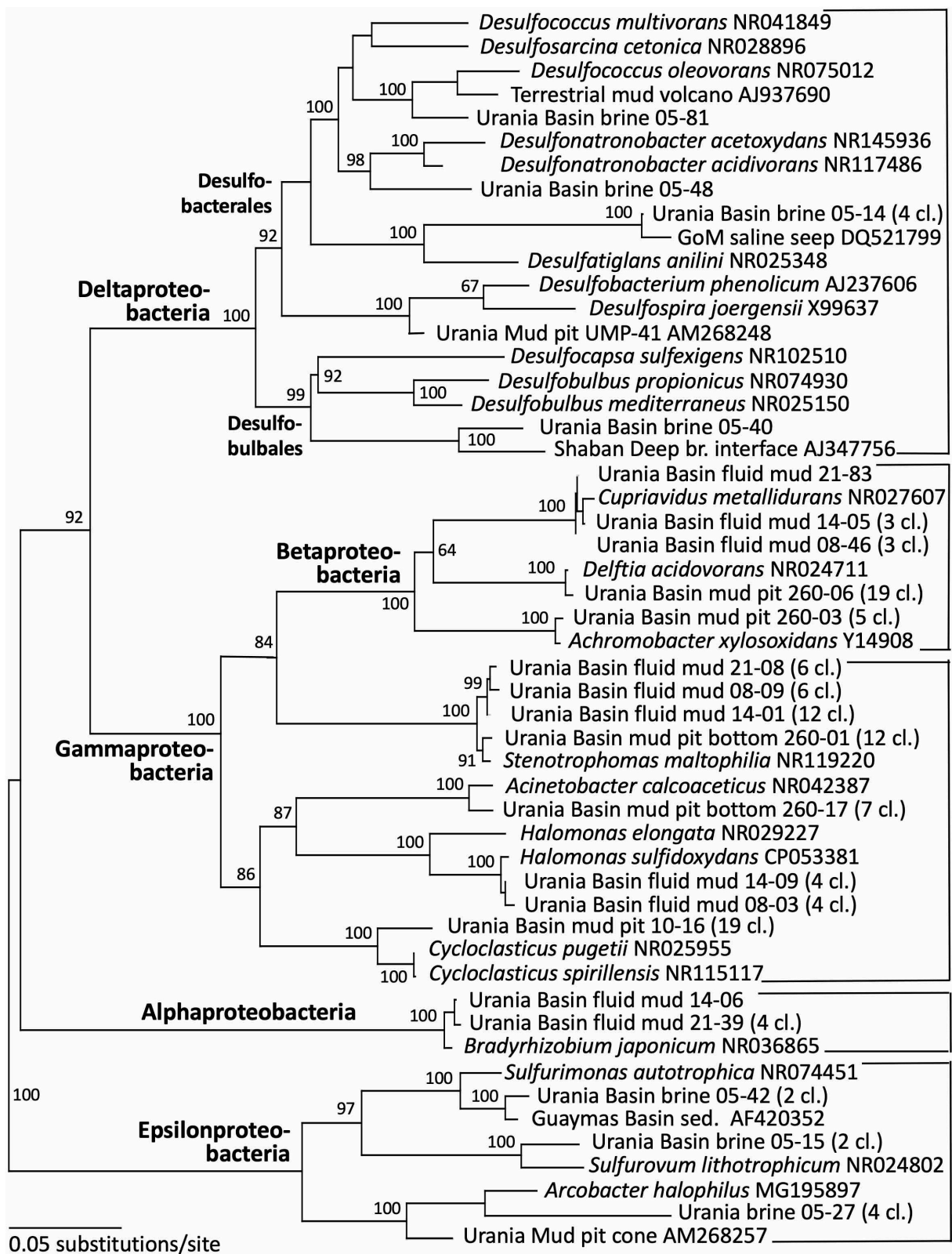
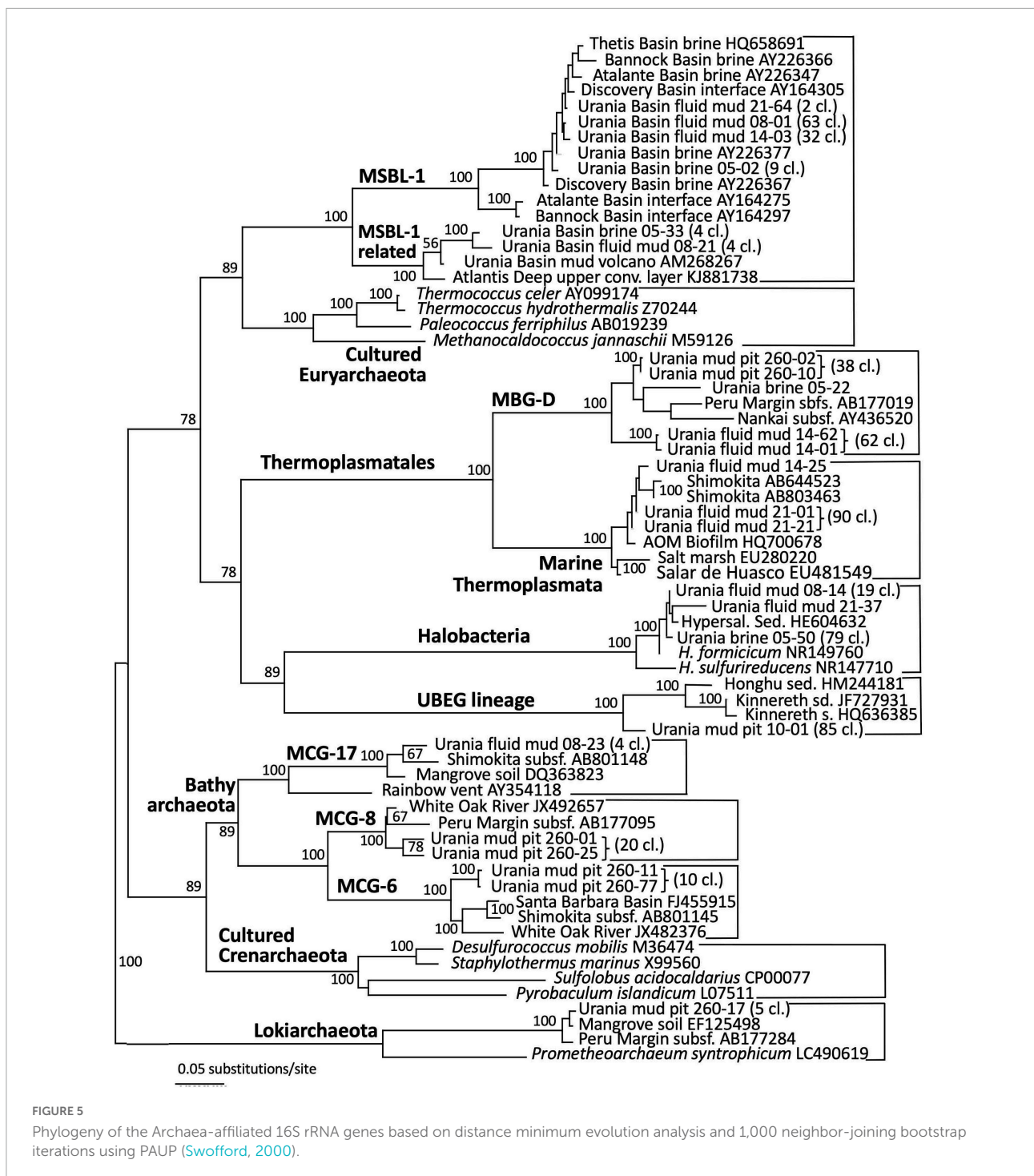


FIGURE 4

Phylogeny of the Proteobacteria-affiliated 16S rRNA genes based on distance minimum evolution analysis and 1,000 neighbor-joining bootstrap iterations using PAUP (Swofford, 2000).



affiliated with genera of the family *Bacillaceae* within the Firmicutes (Figure 6). The upper fluid mud layer yielded a higher proportion of sequences affiliated with moderately halophilic and alkaliphilic *Bacillaceae* related to the genus *Piscibacillus* (Amoozegar et al., 2009), and—a little more distant but within the same monophyletic cluster—halophilic *Bacillaceae* of the genera *Terrilactibacillus*, *Halobacillus*, and

*Pontibacillus* cultured from Urania Basin sediment (Sass et al., 2008). The halophile-related clones co-occur in the upper fluid mud layer with clones related to moderately thermophilic genera *Anoxybacillus* and *Geobacillus*, facultatively anaerobic chemoorganotrophs with a fermentative metabolism (Pikuta et al., 2000; Nazina et al., 2001). In the central and lower fluid mud layer, the halophile-related clones are no

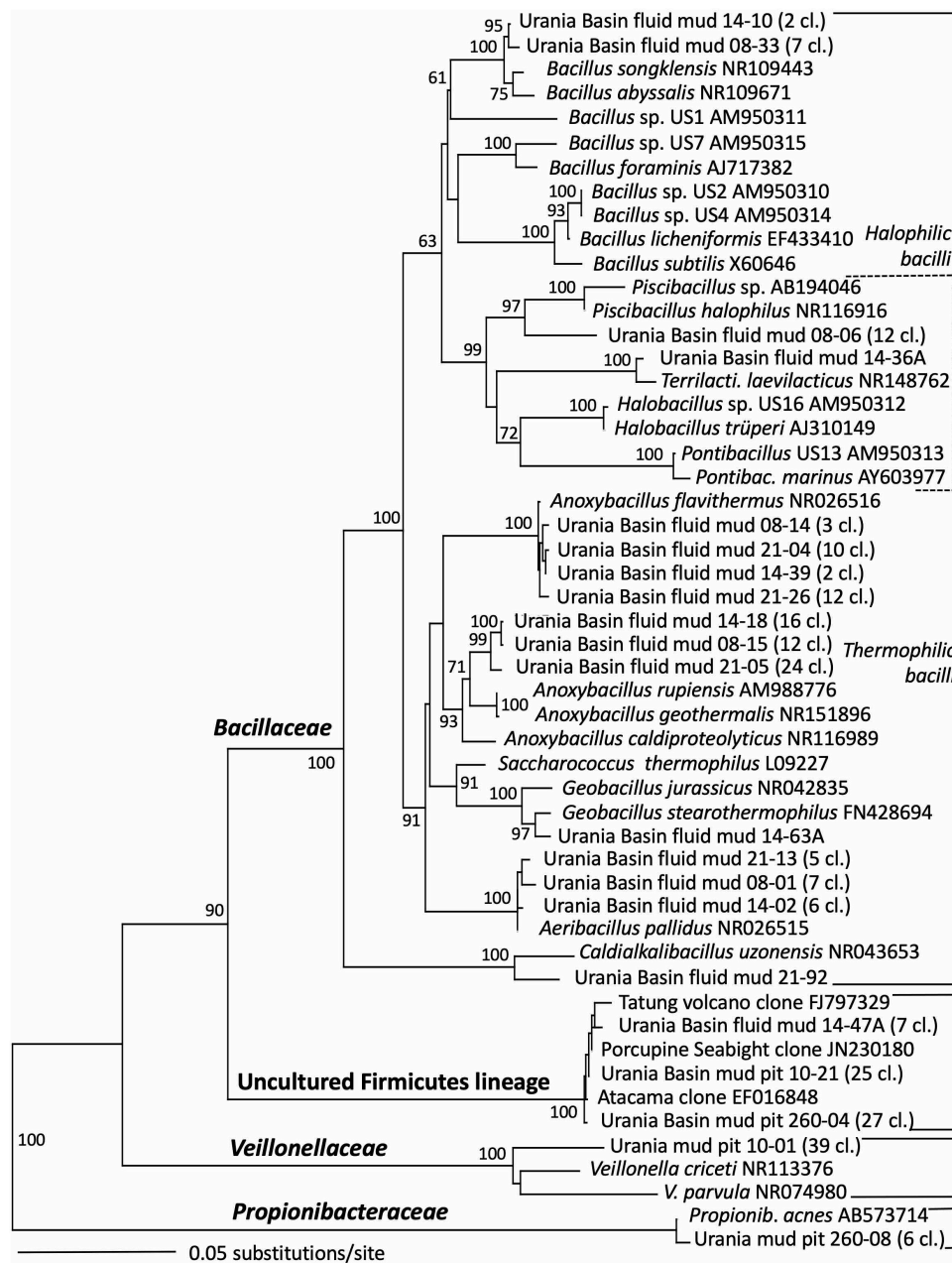


FIGURE 6

Phylogeny of the Firmicutes-affiliated 16S rRNA genes based on distance minimum evolution analysis and 1,000 neighbor-joining bootstrap iterations using PAUP (Swofford, 2000).

longer recovered, and the thermophilic lineages, especially *Anoxybacillus*, predominate. The *Anoxybacillus*-affiliated clones fall into two distinct clusters, one very similar to *Anoxybacillus flavithermus* (Pikuta et al., 2000), whereas the second and more frequently recovered cluster represents a sister lineage to the thermophilic, alkalitolerant, facultatively or strictly anaerobic species *A. rupiensis* and *A. geothermalis* (Filippidou et al., 2016). In contrast to the apparent halophile-thermophile transition, all fluid mud layers yielded almost equal numbers of clones

affiliated with the thermophilic, alkalitolerant, but aerobic species *Geobacillus pallidus*, recently renamed *Aeribacillus pallidus* (Miñana-Galbis et al., 2010). We speculate that these populations represent a terrestrial import that remains detectable due to endospore formation.

Other *Bacillaceae*-affiliated lineages were recovered less frequently, or in only a single layer. Clones related to the marine species *Bacillus songklensis* (Kang et al., 2013) and *Bacillus abyssalis* (You et al., 2013) of the thermophilic,



endospore-forming genus *Bacillus* were recovered from the upper and middle fluid mud horizon. A sequence each from the middle and bottom layer of the mud fluid were affiliated with the thermophilic, heterotrophic, alkalitolerant, and hydrocarbon-degrading species *Geobacillus stearothermophilus* (Nazina et al., 2001) and *Caldalkalibacillus uzonensis* (Zhao et al., 2008), respectively. Further, the central mud layer yielded clones of an uncultured Firmicutes lineage that has consistently close Genbank matches in diverse marine and terrestrial sediment and subsurface environments, and thus appears to represent an evolutionary lineage with a preference for these habitats (Figure 6 and Table 1).

Gamma-, Beta- and Alphaproteobacterial clones formed the second major bacterial group detected in the fluid mud layers, affiliated with the genera *Delftia*, *Cupriavidus*, *Stenotrophomonas*, *Halomonas* and *Bradyrhizobium* (Figure 4 and Table 1). Of these, the genus *Halomonas* (Vreeland et al., 1980) consisting of halotolerant or halophilic, aerobic or facultatively anaerobic isolates from diverse saline environments, occurs specifically in marine environments (Wang and Shao, 2021, and refs. therein). The *Halomonas* clones are closely related to *Halomonas sulfidoxydans*, a highly sulfide-tolerant marine sediment species that oxidizes sulfide aerobically or with nitrate or N<sub>2</sub>O as electron acceptor (Wang and Shao, 2021). *Rhizobium* strains were among the most consistently isolated bacteria from subsurface Mediterranean sapropels (Suess et al., 2004, 2006). In contrast, the hydrocarbon-degrading freshwater and soil bacterium and opportunistic pathogen *Delftia* (Wen et al., 1999; Højgaard et al., 2022), the heavy metal-resistant soil bacterium *Cupriavidus* (Vandamme and Coenye, 2004) and the plant-and human-associated bacterium *Stenotrophomonas* (Ryan et al., 2009) lack marine or subsurface associations. We caution that these sequences were most likely introduced as contaminants during handling of samples, reagents or PCRs, and we are reporting them to provide reference sequences for critical evaluations of microbial community surveys in extreme habitats (Table 1).

The upper and lower mud fluid samples yielded sequences related to *Thermus aquaticus*, a heterotrophic, thermophilic bacterium from thermal springs in Yellowstone National Park that uses several sugars and organic acids as carbon sources (Brock and Freeze, 1969). The genus contains aerobic as well as facultatively anaerobic strains, for example a facultatively anaerobic *Thermus* sp. isolated from South African Gold mines oxidizes organic compounds such as lactate with Fe (III) and/or Mn (IV) as electron acceptors (Kieft et al., 1999). Sequences affiliated with *Thermus* sp. were reported independently at 3,727 m depth in the deep fluid mud of the Urania Basin mud volcano (Yakimov et al., 2007), indicating that this thermophile is indeed a member of this microbial ecosystem.

The archaeal profile of the mud layers shows a transition from predominantly *Halodesulfoarchaeum*- and MSBL-1-affiliated clones in the top layer toward

uncultured Thermoplasmatales lineages, specifically the Thermoprofundales [formerly Marine Benthic Group D (MBG-D)] in the middle layer and a previously undescribed Thermoplasmata lineage in the bottom layer (Figure 4 and Table 1). Extensive Genbank searches and comparisons with published phylogenies specified the phylogenetic position of the Urania Basin clones within the globally distributed and highly diverse Thermoplasmatales. The clones from the middle mud layer were affiliated with the marine sediment subclusters 6, 7, and 8 of the Thermoprofundales (Zhou et al., 2019), indicating tolerance to marine salinity at a minimum. MBG-D sequences have previously been found in abundance in hypersaline, sulfidic methane seep sediments in the Gulf of Mexico (Lloyd et al., 2006), and MBG-D was the major archaeal group in highly sulfidic anoxic sediments of the Salton Sea (Swan et al., 2010). The marine Thermoplasmata clones of the lower mud layer represented a sister lineage of the uncultured DHVE-9/20c-4 clade (Durbin and Teske, 2012) that was originally found at hydrothermal vents (Pagé et al., 2004). Genomic reconstruction has predicted that marine subsurface Thermoplasmatales are involved in detrital matter degradation, as well as acetogenesis (Lloyd et al., 2013; Lazar et al., 2017). The Urania Basin phylotypes were only distantly related to cultured sulfur- and iron-reducing Thermoplasmatales, for example the genera *Thermoplasma*, *Picrophilus*, or *Aciduliprofundum*.

Detection of MSBL1 archaea-related sequences is consistent with previous observations of this group in Urania Basin mud fluids (Yakimov et al., 2007) and the brine lake of the Urania Basin (van der Wielen et al., 2005). The MSBL1 archaea have been detected in many hypersaline environments, such as an endoevaporitic microbial mat (Sørensen et al., 2005), the Bannock, Urania and Thetis brine lakes (Daffonchio et al., 2006; Borin et al., 2009; La Cono et al., 2011), or Tunisian multipond solar salterns (Baati et al., 2008).

### 3.5 Microbial community of the Urania Basin subseafloor sediments

Sequences from the subseafloor sediments (the “mud pit”) differed considerably from those in the overlying fluid mud layers and brine (Figure 2). The uncultured subsurface Firmicutes lineage-affiliated clones that were previously identified in the middle fluid mud layer occurred in larger proportions in both subseafloor sediment samples (Figure 6 and Table 1). The upper gravity core sediments (10–30 cm) yielded sequences affiliated with the fermentative, anaerobic genus *Veillonella*, a widely occurring inhabitant of animal and human intestinal and oral mucosa and an unlikely community member of Urania Basin (van den Bogert et al., 2013). The lower core section (260–280 cmsf) yielded sequences closely related to the fermentative Actinobacterium *Propionibacterium acnes*, reclassified as *Cutibacterium acnes*, a common epibiont



on human skin (Scholz and Kilian, 2016). Due to their likely human origin, we regard the *Veillonella* and *Cutibacterium* sequences as contaminants.

Gammaproteobacterial sequences recovered from the upper gravity core sediments (10–30 cmbsf) were affiliated with the genus *Cycloclasticus*, which utilizes various aromatic hydrocarbons such as naphthalene, phenanthrene, biphenyltoluene, xylene or pyrene as the sole carbon and energy source (Wang et al., 2008; Lai et al., 2012). Cultured representatives of the genus *Cycloclasticus* are strictly aerobic, but their counterparts in anaerobic Urania Basin sediments might represent a related but distinct anaerobic group, compatible with the sequence divergence (ca. 4%) between the cultured strains the Urania Basin clones (Figure 4). Clones from the deeper core section (260–280 cmbsf) were affiliated with the gammaproteobacterial genus *Acinetobacter*, capable of hydrocarbon degradation in hydrocarbon-polluted soils or in oilfield production liquids (Chaîneau et al., 1995; Gong et al., 2012). Sequences of the gamma- and betaproteobacterial genera *Stenotrophomonas* (associated with human clinical samples; Ryan et al., 2009), *Delftia* (an opportunistic emergent pathogen; Højgaard et al., 2022) and *Achromobacter* (described from human sample material; Yabuuchi and Yano, 1981), were found in the deep gravity core sample (260–280 cmbsf) and represent likely contaminants from human contact and handling in the lab.

Archaeal sequence datasets from the upper layers of the gravity core (10–30 cmbsf) were exclusively composed of an uncultured Euryarchaeota Group (Figure 5). This lineage is not phylogenetically close to any known cultured or uncultured archaeal lineage, and the closest uncultured clones were retrieved from lake sediments or Arctic thermal springs. Extensive chimera checks of whole and partial sequences did not uncover any evidence for a mosaic sequence; all sections of the 16S rRNA gene remained ≈15% different from other sequences in Genbank. We called this group Urania Basin Euryarchaeota Group (UBEG).

The deeper gravity core sample (260–280 cmbsf) was dominated by the same uncultured Thermopfundales lineage as in the middle fluid mud layer, and by heterotrophic, anaerobic and presently uncultured Bathyarchaeota of the MCG-6 and MCG-8 subgroups (Kubo et al., 2012). These Bathyarchaeota were distinct from the subgroup MCG-17 clones that were recovered in the upper fluid mud layer (Figure 5). Of these subgroups, MCG-6 has a wide environmental distribution in soil, hot springs, estuarine and marine sediments, whereas MCG-8 and MCG-17 are specifically associated with estuarine and marine sediments (Xiang et al., 2016). Finally, sequences affiliated with the Lokiarchaeota (Spang et al., 2015) were found in smaller numbers. Mixed archaeal communities of Thermoplasmatales, Bathyarchaeota and Lokiarchaeota are characteristic for marine sediments, and were already found in

the first major sequencing survey of marine deep-sea sediments (Vetriani et al., 1999).

## 4 Discussion

The bacterial and archaeal communities of the Urania Basin brine fluids, fluid mud layers and deep sediments show characteristic, habitat-related trends as they change throughout the sample series, after discounting contaminant sequences affiliated to bacteria associated with medical, human, or animal samples (*Achromobacter*, *Delftia*, *Propionibacterium*, *Stenotrophomonas*, *Veillonellaceae*) or terrestrial soil (*Cupriavidus*). Sequences affiliated with sulfur-cycling Epsilon- and Deltaproteobacteria, extremely halophilic KB1 bacteria, and extremely halophilic *Halodesulfoarchaeum* spp. in the brine are replaced by sequences affiliated with diverse Firmicutes, and by halophilic archaea (putatively methanogenic MSBL-1) and Thermoplasmatales in the fluid mud layers. Toward deeper fluid mud layers the Firmicutes change from halophilic (*Piscibacillus*) to thermophilic (*Anoxybacillus*) lineages, and the Archaea change from extreme halophiles (*Halodesulfoarchaeum* and MSBL1) toward subsurface sediment lineages (Thermopfundales and Thermoplasmata). Finally, the deep subsurface sediment below the mud volcano retains sequences related to uncultured Firmicutes, hydrocarbon-degrading Gammaproteobacteria (*Cycloclasticus* spp., *Acinetobacter* spp.), and marine subsurface archaea of the Bathyarchaeota, Thermopfundales, and Lokiarchaeota, plus an unidentified archaeal group that appears only in the upper gravity core sample. Nearly all bacterial and archaeal clones are affiliated to heterotrophic groups, indicating that this downward microbial succession (brine—fluid mud—subsurface sediment) degrades organic substrates and hydrocarbons that are available in the brine, fluid mud layers, and subsurface sediment.

The mud volcano fluids and subsurface sediment microbial communities resemble each other in the presence of gram-positive, sometimes thermophilic, and presumably spore-forming genera and families within the phylum Firmicutes. Thus, the Urania Basin sediment microbiota provide an illustrative example for a “firmicute hotspot,” the previously postulated point sources that distribute endospore-forming, moderately thermophilic Firmicutes across cold marine sediments world-wide (Hubert et al., 2009; Müller et al., 2014; Gittins et al., 2022). These spore-forming bacteria require a certain minimum temperature but cannot grow in deep-sea surficial sediments that are permanently cold. Possible sources include hydrothermal vents, mud volcanoes, and terrestrial sedimentation. Hydrothermal vents have generally very low proportions of gram-positive bacteria, which require lab enrichment for detection (Castro et al., 2021). Terrestrial imports appear likely in some coastal locations (Lee et al., 2005). However, mud volcanoes are the most promising source of

these widespread seafloor thermophiles (Ratray et al., 2022). The Urania Basin Mud volcano, prone to occasional eruptions (Hübner et al., 2003) adds to the database of sources for widely spread gram-positive bacteria in the deep-sea. We speculate further that the carbon and energy source for at least some of these Firmicutes might be petroleum-derived hydrocarbons of deep subsurface origin, since polyaromatic compounds and long-chain alkanes are abundant in the fluidized mud and subseafloor sediment of Urania Basin.

The high contribution of gram-positive Firmicutes in this study stands in contrast to the near-absence of gram-positive bacteria (a single Actinomycete clone) in a previous survey of the Urania mud volcano (Yakimov et al., 2007). These differences might reflect different nucleic acid extraction protocols and PCR targets: chemical extraction of RNA *via* QIAGEN RNA/DNA mini extraction kit followed by reverse transcription to cDNA (Yakimov et al., 2007) vs. bead-beating cell breakage before kit-based DNA extraction and column purification in this study. The mechanical force of bead-beating has very likely increased the recovery of DNA from spore-forming gram-positive bacteria. When bead-beating techniques are used for cell disruption and DNA extraction, gram-positive spore-forming bacteria are recovered from diverse marine sediments (for example, Teske et al., 2002). We note that any DNA-based study might include dead or dormant cells. We also note that the previous study did not involve a nested PCR approach (Yakimov et al., 2007), and thus avoided a likely source of contaminating sequences.

Active sulfur and methane cycling characterize the brine/seawater transition of Urania Basin, where seawater sulfate and subsurface methane overlap (Borin et al., 2009). At present, our data do not rule out active sulfur and methane cycling in the deep brine and the mud volcano fluid. Although the PCR data of this study indicate that sequences affiliated with microbial taxa involved in sulfur cycling appear to be limited to the brine layer, sequences related to sulfur-oxidizing Epsilon- and sulfate-reducing Deltaproteobacteria have been recovered as dominant and active groups by rRNA transcript sequencing from deep fluid mud of Urania Basin (Yakimov et al., 2007). The mud volcano sediments and the overlying brine contain millimolar amounts of methane and sulfate (Borin et al., 2009; Zabel, 2012) and would in principle allow sulfate-dependent methane oxidation to take place. Although anaerobic sulfate-dependent methane-oxidizing archaea (ANME) were not found in this survey, they were detected as a single clone in the deep mud volcano fluid (Yakimov et al., 2007). Few studies have reported on ANME archaea in hypersaline environments. ANME-1 archaea were detected in a moderately briny, sulfidic methane seep in the Gulf of Mexico (Lloyd et al., 2006), and (in low numbers) in the bottom sediments of Orca Basin in the Gulf of Mexico (Nigro et al., 2020). ANME1 and ANME2 archaea were found in the Red Sea seafloor brine lakes (Atlantis II and Discovery Deep), predominantly in a sediment sample

with high total sulfur content (Siam et al., 2012). A consensus reading of the available data indicates that small populations of ANME archaea exist in hypersaline basins and in the Urania Basin mud volcano, but these hard-to-find sequences are not comparable to the global microbial community signature of marine methane seeps, methane/sulfate interfaces or methane-rich subsurface sediments that are dominated by ANME archaea and deltaproteobacterial sulfate reducers (Ruff et al., 2015). Accordingly, we do not observe any evidence of decreasing concentrations of  $^{13}\text{C}$ -isotope enrichments in the methane pool.

Overall, this study highlights the diversity of bacteria and archaea thriving in the extremely harsh conditions of the Urania Basin brine lake and mud volcano. Further activity measurement or culture-based experiments could help understand which microbial metabolisms play a role in the Urania Basin, or how the microbes work together to survive in such an environment.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary material](#).

## Author contributions

CL collected Urania Basin samples, extracted DNA, performed PCR amplification, cloned the PCR amplicons, and submitted them for sequencing, performed the first set of phylogenetic analyses, and wrote the first version of this manuscript. FS, ME, and VH performed and tabulated geochemical analyses of Urania Basin samples, including the methane and ethane analyses. K-UH obtained funding for the DARC LIFE project and for the Expedition to Urania Basin and developed the scientific rationale for sampling site selection. AT advised CL on the sequencing project, prepared the phylogenetic trees in this manuscript, and wrote the second version of the manuscript. All authors revised and finalized the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.1043414/full#supplementary-material>

### SUPPLEMENTARY TABLE 1

Concentration and  $^{13}\text{C}$  stable isotope values of methane and ethane in Urania Basin brine, fluid mud, and subsurface sediment samples.

### SUPPLEMENTARY TABLE 2

Concentrations of hydrocarbons in the subseafloor of the Urania Basin. PAH/HC, portion of polyaromatic hydrocarbons relative to sum of all identified hydrocarbons; Thermal HC/HC, portion of thermally altered hydrocarbons relative to sum of all identified hydrocarbons.

### SUPPLEMENTARY TABLE 3

Similarities of Urania Basin phylotypes and related sequences of pure cultures and environmental phylotypes. Genbank numbers for bacteria and archaea are: OP389385-OP389468 and OP389469-OP389561 in sample 5; OP352680-OP352749 and OP389299-OP389384 in sample 8; OP352780-OP389844 and OP389717-OP389809 in sample 14; OP389562-OP389631 and OP389897-OP389989 in sample 21; OP352539-OP352606, OP389632-OP389716, and OP856603-OP856618 in sample GC-10-30; OP352609-OP352677, OP389811-OP389896, and OP856619-OP856629 for sample GC-260-280.

### SUPPLEMENTARY DATA SHEET 1

Supplementary methods.

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