Characterization of the microbiomes from Salton Sea Trough mud volcanoes, seeps, and enrichment cultures: predominance of H2-autotrophy, H2-producing organotrophy, acetate cycling, and sulfur reduction

Erin Su[1], Anna Dowling[1], Maxim Leshchinskiy[1], Alexandra Turvey[1], Andre Cavalcanti[1], and Edward Crane[1]

[1]Pomona College

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Abstract

The Salton Sea Trough has a range of geothermal features, including mud volcanoes, pots, and seeps that express fluids at temperatures ranging from ambient to 65 to 100 C. The features produce 99% CO2/0.5% CH4 gas of a thermogenic origin and contain hydrothermally-produced petroleum. The mud/clay from the Davis-Schrimpf mud volcanoes has an elemental sulfur (S0) concentration of 317 microM, suggesting that sulfur metabolism should be important in the system. Surprisingly, there has been very little microbiological characterization of these features. Described here are microbial communities, determined by Illumina sequencing of the 515F – 806R 16S rRNA gene fragment, from mud volcanoes/seeps from the Davis-Schrimph seep field and nearby areas with surface feature temperatures from ambient, 65 C, and 100 C. In addition, we have characterized the communities that developed over 1 month at 65 C in enrichment cultures from the 65 C mud volcanoes incubated with a range of electron acceptors (ferrihydrite, S0, SO42-, and S2O32-) and electron donors (tryptone/yeast/casamino acids, crude oil, and CH3CO2-). The 65 C mud and enrichment culture communities were generally predominated by autotrophic H2-oxidizers and organotrophic H2-producers, acetogens, acetotrophs and heterotrophic S0, SO42-, and S2O32- reducers, including many relatives of microbes previously observed in the deep subsurface and from petroleum fields or production waters. While methanogens were present, they were generally at low levels, and few obvious methylotrophs or anaerobic methane oxidizers were detected. Overall, these results provide evidence for a subsurface lithoautotrophic microbial ecosystem (or SliME) in the Salton Sea trough subsurface.

Characterization of the microbiomes from Salton Sea Trough mud volcanoes, seeps, and enrichment cultures: predominance of H_2-autotrophy, H_2-producing organotrophy, acetate cycling and sulfur reduction

Erin Su, Anna V. Dowling, Maxim A. Leshchinskiy, Alexandra K. Turvey, Andre R.O. Cavalcanti, Edward J. Crane III*

Department of Biology, Pomona College, 175 West 6thStreet, Claremont, CA 91711

*To whom correspondence should be addressed

E-mail: ej.crane@pomona.edu; phone 909-607-9634

Abstract The Salton Sea Trough has a range of geothermal features, including mud volcanoes, pots, and seeps that express fluids at temperatures ranging from ambient to 65 to 100°C. The features produce 99% CO_2/0.5% CH_4 gas of a thermogenic origin and contain hydrothermally -produced petroleum. The mud/clay

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Abbreviations used: AMO, anaerobic methane oxidation, NGS, next-generation sequencing, SLiME, subsurface lithoautotrophic microbial environment, TYC, tryptone, yeast and casamino acid containing media

Introduction

The microbial communities that populate the earth's subsurface represent a massive source of both biomass and biodiversity and have only recently begun to be explored. An estimated 10% of the earth's biomass is found in the subsurface, an environment where most of the primary productivity must be due to lithoautotrophy, due to the isolation from the organisms and solar irradiation that drive the photosynthetic primary production on the earth's surface [1]. These environments can range from highly oligotrophic ground water, with relatively little carbon content, to petroleum and natural gas deposits with high carbon content. One obstacle in studying these environments comes from the difficulties in sampling them, which usually requires accessing the environments via either drilling or the use of mine sites. Surface seeps, however, represent a convenient and relatively contamination-free source of fluid from subsurface systems. In these studies, we have taken advantage of surface features that include mud volcanoes and mud seeps to gain an insight into the microbial community present in a hydrothermal hydrocarbon-rich subsurface environment.

The Salton Sea geothermal field is a young (˜16K years) geologic feature characterized by a shallow magmatic intrusion into the Salton Trough sedimentary basin that results from active rifting of the San Andreas and San Jacinto faults in the Brawley seismic zone [2]. The field contains a variety of geothermal features, including deep (>1 km) 350 °C hydrothermal brines and more shallow seep systems. The area in and around the Davis-Schrimpf seep field, located on the southern end of the Salton Sea, contains seeps expressing mud, water and gas at temperatures that range from ambient to 100 °C (Figure 1). The seep fluids, driven by gas comprised of ˜99% CO_2 and 0.5% CH_4, contain petroleum and CH_4 that are believed to result from the hydrothermal alteration of organics [2, 3]. Surprisingly, little microbiological characterization has been performed on the seeps in and around the Davis-Schrimpf field, and the microbial communities they contain have not been described.

We have characterized the microbial communities present in seeps with temperatures that range from ambient to 65 °C to 100 °C, with a focus on the communities from the 65 °C mud volcanoes (while these features are commonly referred to as "mud volcanoes," and we use this terminology here, they are not examples of true mud volcanism, as described in [4]). In addition, we characterized the communities present in enrichment cultures with a range of electron acceptors (Fe^{3+} as ferrihydrite (poorly crystalline Fe^{3+}), S^0, SO_4^{2-}, and $S_2O_3^{2-}$) and electron donors (tryptone/yeast/casamino acids, crude oil and $CH_3CO_2^-$) at 65 °C. Consistent with the CO_2 gas which is emitted at these sites, there was a high proportion of lithoautotropphs, especially H_2-based autotrophs, as well as organisms capable of both acetotrophy and acetogenesis. The original purpose of these experiments was to develop communities capable of anaerobically oxidizing petroleum using sulfur compounds as electron acceptors, and while we do observe organisms likely to be fulfilling that role, we were surprised (perhaps naively) to see that under most of the conditions hydrogen-based autotrophy, acetogens

and acetotrophs seemed to dominate the communities, along with a significant population of sulfur compound reducing organotrophs. Overall, these results provide evidence for a "SliME"- a subsurface lithoautotrophic microbial ecosystem [5, 6] – in the subsurface reservoir that produces the fluids, especially in the case of the 65 °C mud volcano features.

Methods

Sample locations and collection

Samples were collected from three sites at and near the Davis-Schrimpf hydrothermal feature site (Figure 1), including the Davis-Schrimpf mud volcanoes (T = 65 °C, location 33°11'01"N, 115°34'42"W), a nearby collection of hot seeps and mud pots (T = 100 °C, location 33°13'11"N, 115°36'07"W) and an ambient temperature mud pot (fluid temperature = air temperature, at time of sampling 40 °C, location 33°16'33"N, 115°35'37"W). Samples that were to be directly extracted and sequenced were collected in 50 ml centrifuge tubes and placed on ice. Samples for enrichment cultures were injected into sealed 100 ml serum bottles containing anaerobic AB- media (described below).

Enrichment cultures

Each of the 65 °C enrichment cultures contained 25 ml of AB- media (consisting of a solution of 9 mM PIPES buffer, 2 mM K_2HPO_4, 18 mM NH_4Cl, 0.36% w/v NaCl, 1 ml/l of a trace minerals solution, and 3 mg/l rezazurin as a redox indicator, with pH adjusted to 7.0, as described in detail in [7]), an electron donor, either oil (Texas crude oil (Texas Raw Crude International) 2.0 ml), tryptone/yeast/casamino acids, 1% w/v of each (TYC), or acetate (1.0 mM), and an electron acceptor that was either Fe^{3+}, as ferrihydrite (0.5 g, poorly crystalline Fe^{3+}, synthesized as in [8] using the method for 2-line ferrihydrite), $SO_4{}^{2-}$(as Na_2SO_4, 1.0 mM) , S^0 (elemental sulfur, 0.5 g), or $S_2O_3{}^{2-}$ (as $Na_2S_2O_3$, 1.0 mM). Media was made anaerobic by bubbling with N_2. Cultures were initiated by adding collected mud to serum bottles as described above, at a ratio of media to mud of ˜1/4. The cultures were incubated with shaking in water baths at 65 °C for 4 weeks.

Extraction and sequencing

Qiagen's DNeasy PowerSoil Kit was used to extract DNA according to the kit procedure without optional incubations. Duplicate samples were taken from the environmental samples, and for enrichment cultures, each condition was performed in triplicate, with a single sample for sequencing taken from each of the cultures. Each sample (environmental and enrichment cultures) used approximately 1 to 2 ml mud/sediment.

Molecular Research LP (https://www.mrdnalab.com/) performed the sequencing of the extracted DNA from the samples using the 515F and 806R primers [9] and the following protocol: The HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used for PCR (barcode on forward primer) with the following cycle: 3 minutes at 94°C, then by 30 cycles of 30 seconds at 94°C, 40 seconds at 53°C and 1 minute at 72°C, and followed by a final elongation step of 5 minutes at 72°C. PCR products were pooled and used to create an Illumina DNA library, then sequenced using Illumina MiSeq v3 2 × 300 bp sequencing according to manufacturer guidelines (Illumina, San Diego, CA, USA). All sequences obtained in this project are available at GenBank under BioProject PRJNA891239.

Sequence Analysis

Reads were processed using Qiime2 [10]. Reads were imported into qiime and we used the cutadapt qiime plugin [11] to filter reads without the primer sequences in the forward and reverse reads.

The reads were then processed using DADA2 [12] (using the q2-dada2 qiime plugin) to generate a table of unique amplicon sequence variants (ASV) and their counts per sample. Taxonomy for each ASV was determined using the q2-feature-classifier plugin [13] classify-consensus-vsearch taxonomy classifier against the Silva database version 138 [14].

Samples were rarefied to 19,000 based on the number of reads of the sample with fewer reads, and alpha and beta diversity measures were calculated using qiime's diversity plugin. For each sample, we calculated α-

richness (number of different ASVs, observed features), diversity (Shannon Entropy), phylogenetic diversity (Faith's phylogenetic diversity metric), and evenness (Pielou Evenness index). To estimate the differences between samples we used the Bray-Curtis index.

All the commands used in the qiime2 analyses are available in the paper's GitHub repository: https://github.com/aroc110/Su_et_al_2022.

S^0 concentrations in 65 C mud volcano clay/fluid and GCMS analysis of oil from enrichment cultures

S^0 concentrations were determined by HPLC, using the method described in [15]. To test for enrichment cultures' ability to oxidize both straight chain and aromatic hydrocarbons, crude oil (2 ml, Texas Raw Crude International) was added, along with a trace amount of TYC (0.01% w/v), to 25 ml of the anaerobic AB-media described above in a sealed 100 ml serum bottle, with either no acceptor, S^0, or Fe^{3+}, and inoculated as described above. A sterile control with no mud inoculation was also prepared. The resulting culture was grown at 65°C for 4 weeks. Following incubation, ˜ 0.5 mL of the oil layer was extracted and dissolved in 0.5 mL hexane. Any aqueous layer was removed and the solution was filtered through cotton, and the resultant material analyzed with an Agilent 6890 Series GCMS instrument.

Results and Discussion

While 16S rRNA amplicon Illumina sequencing is known to produce results with some degree of bias, recent work comparing it with high-throughput qPCR [16] – generally regarded as the gold standard for quantification of microbial species in a community – and metagenomic sequencing [17] have shown that the methods produce very similar results [16]. While HT qPCR has the advantage of being able to more readily differentiate between closely related species and metagenomic sequencing tends to detect more species in a sample, 16S rRNA amplicon sequencing can detect organisms missed by qPCR. In any case, the three methods generally show surprisingly good agreement, with 16S rRNA NGS being especially effective in determining the overall makeup of the community, albeit with lower resolution and sensitivity when compared to other methods [16, 18].

This means that the methodology employed here, despite its shortcomings, allows us to determine the predominant microbial metabolic types present and gives us an idea of the relative amount of each guild in the community.

Main energy-conserving metabolisms present

To obtain an overview of the main metabolic guilds present in the 65 °C mud and in the 65 °C enrichment cultures we have attempted to determine the most likely metabolism for the microbes that were detected at high levels in each case, as shown in Scheme 1, Figure 2 and Table 1 (in supplemental results). While many identifications could only be resolved at the family level, we have chosen the most likely energy-conserving metabolic type for each organism based on the available evidence in the literature. The identified organisms were generally present at a level of at least 1% in at least one of the samples, except in the cases of methanotrophs and sulfur-dependent autotrophs, which were generally consistently present at very low levels. For facultative organisms and/or organisms that could fall under multiple categories – such as *Thermoacetogenium,* capable of both acetogenesis and acetotrophy [19] – we have mentioned known alternate metabolisms in the text or in Scheme 1, where many of the organisms are shown for multiple reactions.

It is highly likely, however, that several of the organisms discussed have multiple alternative energy conserving metabolisms that are not known. Given these limitations, and the limitations of community analysis by 16S rRNA gene sequencing, there is likely to be a high degree of error in the absolute percentages of the main metabolisms present. While these results are at a more qualitative than quantitative level, the main metabolic guilds present support the characterization of the mud volcano environment (and the subsurface fluids expressed by these features) as being dominated by H_2-driven autotrophy, with a significant contribution from acetogens and acetotrophs and sulfur compound-reducing heteroorganotrophs, as shown in Figure 2 and Scheme 1.

General compositions of communities from 65 °C mud volcanoes and enrichment cultures

The organisms in the observed communities were generally consistent with communities previously observed in thermophilic environments, especially subsurface communities with a significant hydrocarbon content [20-23], consistent with the characterization of the mud/clay from these features as petroleum rich, and the emitted gas as 99% CO_2 and 0.5% CH_4 [24]. The presence of organoheterotrophs previously observed in petroleum fields, production waters and underground natural gas storage reservoirs, including *Pseudothermotoga, Thermosipho, Desulfotomaculales* , a Firmicute in the genus/family SCADC1-2-3, *Desulforhabdus, Thermodesulfovibrio* and *Fervidobacterium* [25-32], confirm that the environment is petroleum rich, and suggest that hydrocarbon metabolism may play a significant role in this environment. Both the environmental samples and enrichment cultures had significant populations of autotrophic H_2-oxidizers, varying from a low of 1.4% in S^0/TYC enrichments to a high of 58% in the 65 °C mud volcano community, with most enrichments showing high levels of this guild (Figure 2). Autotrophic acetogens, acetotrophs, and acetogenic and CO_2-producing organoheterotrophs capable of S^0, SO_4^{2-}, $S_2O_3^{2-}$, Fe^{3+} and Mn^{2+}-based respiration also made up a significant part of the communities (Figure 2). Based on the measured S^0 concentration of 317 µM in the 65 °C mud volcano mud/clay, the large % of sulfur and sulfur compound-reducing organisms (see below) was not surprising.

Based on these broad observations, the community is consistent with a hydrogen-dependent subsurface lithoautotrophic microbial environment (SLiME, [33-35]), with lower levels of methanogenesis and/or anaerobic methane oxidation than observed in some previous cases [36, 37], and with a large component of sulfur reduction. A basic scheme describing the hydrogen, sulfur and carbon cycling, with some representative organisms involved in that cycling is shown in Scheme 1.

The low levels of methanogens (except in the TYC-containing enrichments), with the absence of known anaerobic methane oxidizers (AMOs), suggests that a significant supply of alternate electron acceptors such as sulfate are present, although given the relatively high methane concentrations observed in the emitted gas, the absence of known AMO organisms is a bit surprising. Few aerobic methane oxidizers were observed as well. In one of the 65 °C mud samples a species of*Crenothrix* [38] was observed at 1.8%, however this organism was not observed at significant levels in the enrichments. In the 65 °C S^0/oil enrichment *Methylorubrum* [39] was present in two of the samples but at less than 0.5%. The samples from the ambient seep contained *Methylohalobiaceae* [40] at ˜0.2% (see below mud comparisons). These communities are quite distinct from those observed in a more oligotrophic subsurface environment, in which methanogenesis, AMOs and sulfur oxidizing denitrifiers were the predominant metabolic guilds [41].

The large fraction of organisms falling under the metabolic cstegory of "other" identified for the Fe^{3+}/acetate enrichment samples compared with that of the other conditions (Figure 2), is comprised mostly of an uncharacterized bacteria that was found at lower levels throughout the other cultures and in one of the 65 °C mud samples. This bacteria corresponds to 47% of the reads in the Fe^{3+}/acetate enrichment cultures. A blastn search at GenBank showed a perfect match (100% identity throughout the full length of the read) to accession number KU073919.1, which corresponds to a read obtained from an oil field.

Based on a PCoA plot of the 65 °C mud and enrichment cultures (Figure 3), it is fairly clear that in the enrichment cultures the identity of the electron donor has the strongest effect on community composition, with rich media (TYC) enrichments strongly separated from the acetate and oil enrichments, and oil and acetate enrichment communities overlapping. PCoA analysis of the communities that developed with each electron donor do show, however, that electron acceptor does have a significant effect on community makeup (Figure 4A,B,C).

While GC/MS analysis of the oil enrichment cultures showed no observable change in the peak ratios of the crude oil over the month-long incubation in the presence of S^0 or ferrihydrite as electron acceptors, or in an enrichment with no acceptor present (supplemental material, Figure 1), the dissimilarities observed between the oil and other enrichment cultures indicate that a community adapted to the presence of crude oil had developed (Figure 3). Presumably some oxidation of crude oil was taking place, although not at a level that

was detectable over the relatively short time frame of the experiment.

H_2-dependent autotrophs

In the 65 °C mud sample and most of the enrichments *Caldimicrobium* (family *Thermodesulfobacteriaceae*) [42, 43] and a *Hydrogenothermus* [44, 45] were the predominant H_2-utilizing autotrophs, comprising 56% of the community in the 65 °C mud sample, with generally lower but significant amounts across the enrichment cultures (Figure 2, Table 1). A *Caldimicrobium* species capable of autotrophic growth via sulfur disproportionation has been discovered [46], however our analysis does not have sufficient resolution to identify the *Caldimicrobium* to the species level. A *Hydrogenobacter* species (family Aquifaceae) [44, 47] was generally present in low amounts, except in the $S_2O_3^{2-}$/acetate enrichments, where it was ˜5% of the community. As described below, facultatively autotrophic H_2 oxidizers such as *Desulforudis* and *Thermodesulfovibrio* were also present, although not included in the predicted percentage of H_2-dependent autotrophs. The acetogenic autotroph *Moorella* [48, 49] was present in the 65 °C mud and the oil enrichment cultures, at levels of ˜3-20% (excepting the S^0/oil enrichments).

Methanogenic H_2-dependent autotrophs

Methanogens were generally present at low levels, with *Methanothermobacter* , *Methanomassiliicoccalaes* [50, 51] and a species from the family Methanothermobaciaceae found at low levels, except in the enrichment cultures containing TYC and Fe^{3+}, $S_2O_3^{2-}$ or SO_4^{2-}, where they made up between 15-25% of the community.

Acetogens and acetotrophs

Organisms capable of acetogenesis by both autotrophic and chemotrophic metabolisms were found at high levels in the community. The acetogenic fermenter *Acetothermia* [29, 52, 53] could serve as one of the carbon sources for the acetotrophic community members, and when combined with the observed high levels of the acetogenic autotroph *Moorella* , they make up ˜4% of the 65 °C mud. Other acetogens present in significant amounts include the S^0-reducing *Thermosipho [22, 54]* and H_2-generating *Fervidobacterium* [55] (both from the family Fervidobacteriaceae), and the H_2 and acetate-producing *Desulfofundulus* (family *Desulfotomaculales*) [56-58]. Other organisms from the 65 °C sample and enrichment cultures capable of acetotrophy included the sulfate reducing *Thermodesulforhabdus* (family Thermodesulforhabdaceae) and *Thermacetogenium* (family Thermacetogeniaceae) (Scheme 1, Table 1). It is interesting to note that an organism from the uncultured candidate family *Hydrothermae* [59, 60] was found at levels of ˜1-8% in enrichments containing acetate and sulfate or thiosulfate (Table 1), suggesting a possible function for the these uncharacterized organisms as acetotrophic sulfate and thiosulfate reducers.

H_2-generating organoheterotrophs

Relatively high levels of organisms likely to be capable of H_2 generation were observed in all samples, providing support for the high proportion of H_2-dependent autotrophs in the samples. Bacteria in the genus *Pseudothermotoga* (family Thermotogaceae) [61-63] are capable of using protons as electron acceptors (*Pseudothermotoga* includes many species previously placed in the genus *Thermotoga*). Other organisms from the communities that were capable of H_2 production included *Thermosipho* and *Fervidobacterium* (from the family Fervidobacteriaceae) [32], *Thermogutta* (family *Pirellulaceae*) and *Desulfofundulus* (family Desulfotomaculales). In our classification of likely metabolisms we placed the archaeon *Thermococcus* , found at high levels throughout the enrichment cultures, in the category of S^0, $S_2O_3^{2-}$, SO_4^{2-}-reducing organotrophs. They are, however, capable of H_2 production, especially in the absence of S^0 [64, 65].

Sulfate, thiosulfate and sulfur-reducing organoheterotrophs

Organisms capable of growing via the dissimilatory reduction of sulfur, thiosulfate and sulfur made up a significant proportion of both the 65 °C sample and enrichment cultures (Table 1, Figure 2), an expected result given the 317 µM concentration of elemental sulfur in the mud volcano sample. The oil-containing enrichments consistently had the highest proportion of these organisms. The acetogenic and hydrogen-generating *Thermosipho* (family *Fervidobacteriaceae*) is likely capable of S^0 or $S_2O_3^{2-}$ reduction as well [32,

66-68]. In addition, while predictions made from the enrichment cultures are hypothetical, the high levels of *Thermodesulforhabdus* observed in acetate and oil enrichments with S^0, $S_2O_3^{2-}$ and SO_4^{2-} as acceptors suggest that the species in question may be able to reduce S^0 and $S_2O_3^{2-}$, unlike the previously observed species, which is only able to reduce SO_4^{2-} [27]. Thermoanaerobacteraceae were found at high levels in TYC enrichment cultures containing S^0, SO_4^{2-} and $S_2O_3^{2-}$, consistent with the observation that members of this family are generally S^0 and $S_2O_3^{2-}$ reducers [69-71]. A member of the Thermaerobacteraceae, a family of mostly aerobic organisms, was found at 6.7% in one of the three S^0/oil enrichment cultures and at low but consistent levels throughout the other cultures, consistent with the finding of anaerobic examples within this family [72, 73]. While the finding of the Thermaerobacteraceae observed here in an enrichment containing S^0 suggests it may be capable of sulfur reduction, we have classified it as a general organotroph for a lack of direct evidence of this metabolism.

Generally, the S^0-containing enrichments had the most consistently high proportion of sulfur compound reducing organisms (Figure 2, Supplementary Table 1). These organisms were present in significant amounts throughout the enrichment cultures, with the lowest % observed in the Fe^{3+}/TYC enrichments, which contained ˜13% sulfur reducers – still a significant amount. The acetate-containing enrichments had the most consistently high levels of sulfur compound reducing organisms, consistent with our choice of this electron donor as one likely to select for anaerobic respirers. There was little or no effect of enrichment with S^0 on the low levels of identified sulfur-oxidizing autotrophs across all conditions, with the majority of the autotrophs across all conditions remaining H_2-dependent.

Overall, members of the *Thermococcaceae, Archaeoglobacea [74], Thermodesulfovibrio* [29, 75], and *Thermodesulforhabdus*[27, 76] make up a large portion of the 65 °C mud community and the enrichment cultures (Table 1). A species of *Thermus* (family *Thermaceae*), generally considered to be aerobic, was observed throughout the 65 °C samples and at ˜5-10% in the 65 °C mud and ˜25% in one of the three S^0/oil enrichment cultures, consistent with the observation that several strains of *Thermus* are capable of growing anaerobically using S^0 and other electron acceptors [77, 78]. As mentioned above, few organisms capable of growing via sulfur compound oxidation were observed in the community.*Hydrogenobacter* , an *Aquificae* able to grow autotrophically via H_2 or sulfur [44], was seen at low levels in the 65 °C sample, and at 20% in one of the S^0/oil enrichment samples.

Comparison of ambient, 65 °C and 100 °C mud/seep microbial communities

Figure 5 shows the taxonomic composition of the 3 different temperature seeps, resolved at the kingdom, phylum and family level. As would be expected, the 100 °C sample had the highest proportion of archaea of the three environments, however, it was somewhat surprising that the majority of the detected community was bacterial. Not surprisingly, the ambient mud sample had the highest richness (observed features) and Faith's phylogenetic diversity indices (Figure 6), although not the highest diversity (Shannon) as evenness was elevated in the 100 °C environmental sample. The enrichment cultures, selected by donor, had relatively similar alpha richness, as they were similar across the four measures, although the S^0-containing enrichments had the lowest diversity (Shannon).

Ambient temperature seep community

In the ambient temperature mud there is evidence for a sulfur-cycling community. The facultatively chemolithoautotrophic *Deferribacter*was the predominant organism, present at ˜35% (Table 1, supplementary material). While the *Deferribacter* genus is metabolically diverse, *Deferribacter* species tend to be facultative autotrophs capable of using S^0 and Fe^{3+} as electron acceptors during organotrophic growth [79-82]. Organotrophic *Bacteroidota* made up ˜30% of the community, consisting mainly of a Bacteroidetes_VC2.1_Bac22, a newly discovered sulfur-reducer common to anoxic and sulfidic environments [83], and a *Tangfeifania*species [84].

Desulfobacterota made up ˜10% of the community, compared to the levels of 40% in the 65 °C sample and 2-14% in the 100 °C sample. The main *Desulfobacterota* present appeared to be sulfate reducers, including *Desulfosarcinaceae* , common to water-flooded petroleum reservoirs, at 4-7% [85],*Syntrophobacter*

, at ˜1%, and the facultatively chemolithoautotrophic *Desulfovermiculus* at 0.5% [86]. There is evidence for the potential for sulfur-dependent autotrophy, with the γ-protebacterial *Guyparkeria* (a member of the Thioalkalibacteraceae family) [87] and a member of the*Chloroflexi* present at 3 and 2%, respectively. The strictly anaerobic lithoheterotrophic archaea *Halanaeroarchaeum* , which is capable of sulfur-dependent H_2 or formate oxidation [88], was present at ˜1%. *Pateascibateri* , proposed to be anaerobic fermenters in the newly defined superphylum Pateascibateri that were previously associated with oligotrophic conditions [89], were present at 2-3%. Methanogenic members of the Euryarchaeota were present at 0.3-0.6%.

100 °C seep community

There was significant variation between the two samples from the 100 °C environment, almost certainly due to the low biomass and subsequent low levels of DNA likely to be present in this extreme environment. For this reason, the following summary only includes organisms present in both samples. The predominant organisms were several members of the Crenarchaeota, including a member of the versatile anaerobic respirer*Pyrobaculum* (21-32%) [7, 90-93], and the fermenters*Ignisphaera* (1-6%) [94] and the sulfur-dependent*Thermofilum* (0.5-0.8 %) [95]. At the phylum level, Firmicutes were 12-35% of the sample and Desulfobacterota were 2-14%. Proteobacteria, which were mostly γ-proteobacteria, were 10-12%, and the Bacteroidota, and Actinobacteriota were present at 6-12% and 3-9%, respectively.

Comparison to previously characterized subsurface environments

From a survey of subsurface environments, or fluids from active geologic features, it becomes quite clear that while autotrophy and one or two carbon compound cycling are quite common, the main microbially-driven element cycling and the metabolic guilds present differ markedly, based on the fluid environment and geochemistry. Even within environments that are generally similar – for example, hot petroleum reservoirs – the composition of the communities, in terms of the main energy-conserving metabolic guilds present and their relative amounts, vary significantly.

High temperature petroleum-rich environments

While a hot (65 °C) water-flooded off-shore petroleum field in China yielded many individual organisms similar to those in this study, a very different metabolic profile was observed. The community in this case was predominated by methanogens, fermenters and sulfate reducers [96]. It should be noted, however, that while their survey was extensive (88 phylotypes found, 60 bacterial and 28 archaeal), microbes were identified by cloning of 16S rRNA genes rather than NGS, which could result in different biases. From a hot (95 °C) oil field reservoir at Terra Nova, Canada, a community made up of methanogenic and S^0 and sulfate reducing archaea, as well as sulfate and sulfur reducing bacteria and hydrocarbon-degrading organisms were observed, including *Thermosipho* , observed here [97]. The community from the 95 °C Uzen oil field in Kazakhstan had several similarities to the 65 °C mud volcano samples. In one of the injection water samples 55.7% of the microbes observed were the autotrophic Desulfobacterota, compared to 37% in the 65 °C mud volcanos described here [22], and *Thermodesulfohabdus* and *Thermosipho*were well represented, as they were in our samples. The injection waters typically had much higher concentrations of methanogens, however, with *Methanothermococcus* typically at levels of ˜20%, and as high as 71.3%, compared to <0.01% methanogens observed in the 65 °C mud volcanos.

Mud volcano systems

A range of mud volcanoes from across the world show that these sites are often predominated by methanogens and methanotrophs as well as sulfur-dependent chemolithoautotrophs, differing significantly from the communities described in these studies. Mud volcanoes from Azerbaijan, including samples with temperatures close to 65 °C, were dominated by sulfur cycling, and while a *Desulfutomaculia* species related to the *Desulfotomacules SCADC-1_2_3* species observed here was a likely sulfate reducer in the system, other observed sulfate reducers (members of the *Desulfobacteraceae* and *Desulfobulbaceae*) and the presence of a significant sulfur-oxidizing guild differed quite markedly from our results [98]. A lower temperature (16 °C) mud volcano from Crimea contained a community of sulfide oxidizers and aerobic and anaerobic metha-

notrophs, both of which were conspicuously absent from the 65 °C samples described here [99]. Microbial communities in fluids from mud volcanoes of the Beciu area in Romania were dominated by methanogens, anaerobic methane oxidizers, and S^0 and sulfate reducers [100].

Low to high temperature ground water and spring/vent systems

In terms of the relative abundances of energy-conserving metabolic guilds, the community from the CO_2-saturated (non-hydrothermal) groundwater fluids of Crystal Geyser in Utah, USA were similar to the 65 °C mud volcano communities described here, except for the high proportion of sulfur oxidation predicted to take place in the groundwater fluids [34]. Other than the CO_2saturation, this site was quite different from the 65 °C mud volcanos, being oligotrophic and low temperature, and there was very little similarity between the organisms identified at the sites, as would be expected. Based on their analysis of the metagenome, however, the authors proposed that high degrees of H_2-based autotrophy, acetogenesis and acetotrophy (as well as lactate and formate cycling) and moderate amounts of dissimilatory sulfur reduction were features of the communities. Little or no methanogenesis or methanotrophy was predicted. From this comparison, which differs only in the inclusion of sulfur oxidizers, it is obvious that the CO_2 saturation at that site and the site described here exert a major control on the community makeup, which is not unexpected.

Contrasting with the above, while the microbial ecosystem in oligotrophic, deep, fluid-filled fractures in the Witwatersrand Basin, South Africa was predicted to be a SLiME, the autotrophy was shown to be driven by 4 genera of β-proteobacterial sulfur-dependent autotrophic denitrifiers, with sulfate reducers, anaerobic methane oxidizers, and methanogens, each of which was present at a few percent [41]. Highly oligotrophic deep subseafloor sediments, with their very low density of microbes, tend to have populations made up largely of the bacteria in the Chloroflexi, Gammaproteobacterial, and Planctomycetes phyla, with the majority of the archaea in the phyla Crenarchaeota, with the remaining archaea being mostly in the Euryarchaeota and Thaumarchaeota [101].

A survey of hydrothermal vent fluids in Yellowstone Lake high in CO_2, CH_4 and H_2S found a community likely to have high levels of hydrogenotrophs, and similarly to our results few or no methanotrophs were observed, despite significant levels of methane [102]. Hydrogenotrophic hot springs communities found in Yellowstone at Roadside West Hot Springs, a neutral hot spring at 44-68 °C, were proposed based on geochemistry and observed H_2 consumption and isolation of H_2-dependent autotrophic cells [103]. Despite the similar temperatures, the identity of the organisms present appeared to be very different from that of the 65 °C samples described here, with hydrogenotrophy in this case being driven by an organism related to *Thermodesulfobacterium,* and *Thermoflexus* and *Thermofilum* species.

Conclusions

Mud volcanoes and seeps located in the Salton Trough geothermal field in southern California deliver fluids and clay/mud from subsurface sources. The 65 °C fluids ejected from a hot subsurface clay/petroleum source, accompanied by CO_2 and CH_4-containing gas, contain a community of microbes dominated by H_2-based autotrophy, acetate cycling, and sulfur-compound reducing and H_2 producing organotrophs, as determined by analysis of 16S rRNA amplicon sequencing. These results are consistent with a subsurface lithoautotrophic environment, and are similar to previously characterized subsurface communities, although the communities described here have much lower levels of sulfur-dependent autotrophy, methanogenesis and methane oxidation than many other subsurface communities. Dissimilarities between enrichment culture communities were strongly driven by the identity of the added electron donor, although community composition for each electron donor type was also driven by electron acceptor.

Figure 1 A, B) Location of sampling sites, southern California, USA, near the Salton Sea and located in the Salton Sea geothermal field in the Salton Trough **C)** 65 °C mud volcanoes at Davis-Schrimpf field, **D)** actively degassing fumarole/gryphon feature from Davis-Schrimpf field (100 ml serum bottle for size comparison), **E)** 100 °C seeps/mud pots, **F)** ambient temperature seeps, **G)** active fluid-producing feature from ambient seeps, 100 ml Kimax bottle cap for size comparison

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Figure 2 Percentages of the prokaryotic microbial population with the identified major carbon and sulfur cycling energy-conserving metabolic pathways observed in the 65 °C mud volcano sample and in 4 week anaerobic enrichment cultures from that source, with the electron donor/acceptor pairs shown, with the pathways predicted as described in the text.

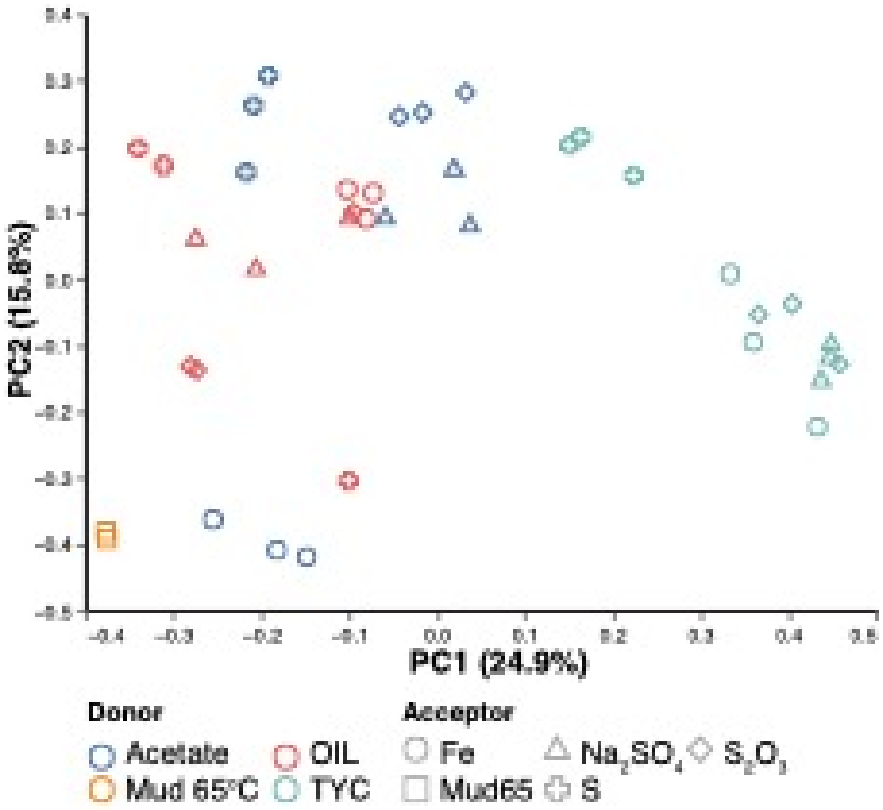

Figure 3 PCoA plot based on Bray-Curtis dissimilarity of microbial communities, determined by 16S rRNA gene profiling via Illumina sequencing, in 65 °C mud and 65 °C anaerobic enrichment cultures grown for 1 month, with varying acceptors and donors

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Figure 4 PCoA plots based on Bray-Curtis dissimilarity of microbial communities, determined by 16S rRNA gene profiling via Illumina sequencing, in 65 °C anaerobic enrichment cultures grown for 1 month, using **A)** oil, **B)** TYC and **C)** acetate as electron donors and the electron acceptors shown (Fe = ferrihydrite, Na2SO4 = SO_4^{2-}, S = S^0 and S2O3 = $S_2O_3^{2-}$).

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Figure 5 Relative abundance of prokaryotes at the kingdom, phylum, and family level from ambient, 65 °C and 100 °C sites at or near the Davis-Schrimpf geothermal field, as determined by 16S rRNA gene profiling via Illumina next generation sequencing.

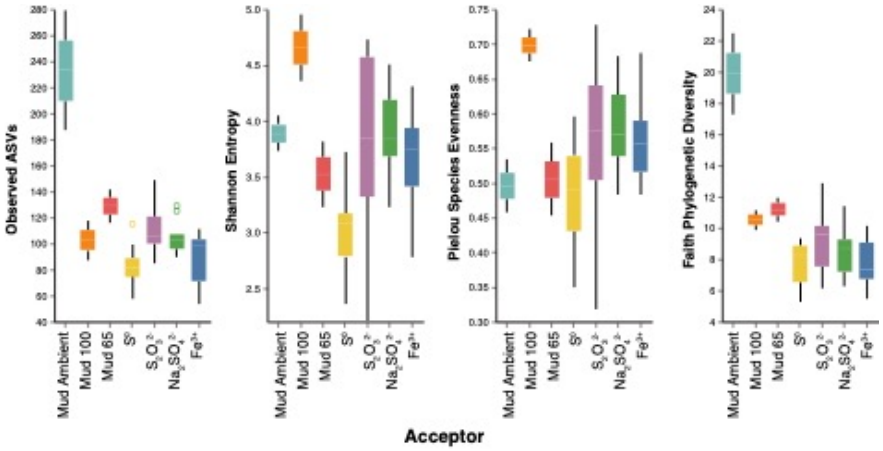

Figure 6 Alpha diversities of prokaryotic communities from ambient, 65 °C and 100 °C sites and from 65 °C enrichment cultures (listed by electron acceptor), estimated using Shannon, species richness (observed features), phylogenetic diversity (Faith's PD) and Pielou's evenness indices.

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Scheme 1 Simplified diagram of major organo- and autotrophic pathways observed in the 65 °C mud volcanoes and/or in 65 °C anaerobic enrichment cultures from that source grown on a range of acceptors and donors, showing organisms present (usually at the genus level) predicted to be capable of that metabolism

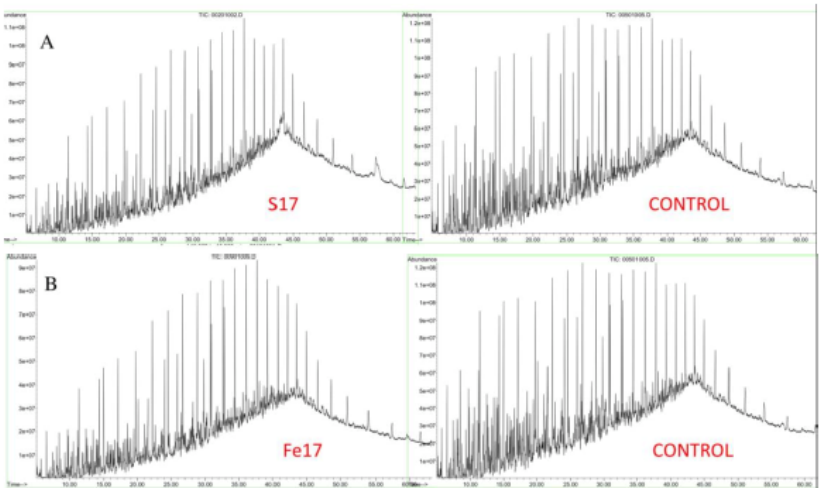
Supplementary Table 1: Relative abundances (%) of selected families and guilds in the 65 °C mud and different enrichment cultures.*

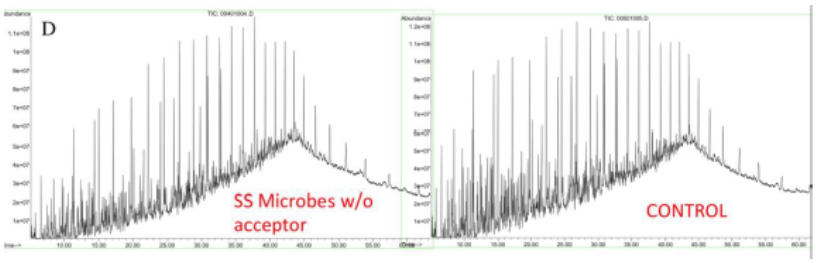
		Mud	**TYC**	**TYC**	**TYC**	**TYC**	**Ace**
Metabolism	**References**	65	S^0	$S_2O_3^{2-}$	$Na_2SO_4^{2-}$	Fe^{3+}	S^0
H2-dep autotrophy		60.36	0.90	5.43	1.56	2.19	20.77
f__Aquificaceae	[44, 47]	0.30	0.02	0.01	0.01	0.01	0.02
f__Hydrogenothermaceae	[45, 104-106]	20.83	0.17	0.08	0.07	0.10	1.51
f__Moorellaceae	[48, 49]	2.09	0.31	2.10	0.35	0.89	0.14
f__Thermodesulfobacteriaceae	[42, 43, 46]	37.14	0.41	3.23	1.13	1.18	19.10
H2-dep autotrophy, methanogenic		0.00	0.79	19.80	20.56	25.94	0.09
f__Methanomassiliicoccaceae	[29, 50, 51, 107]	0.00	0.02	8.47	3.55	9.72	0.02
f__Methanothermobacteriaceae	[96, 108, 109]	0.00	0.77	11.33	17.02	16.22	0.08
Organotrophs, including acetotrophs		10.27	23.40	22.04	19.64	19.35	2.08
f_Acetothermiia	[29, 52, 53]	1.90	0.05	0.20	0.63	0.27	0.06
f_Aminicenantales	[110, 111]	0.69	0.04	6.54	3.39	1.47	0.18
f_Bacillaceae	[112]	1.17	6.60	6.17	2.60	11.05	0.86
f_Calditrichaceae	[113]	0.67	0.00	0.00	0.00	0.00	0.04
f_Caldicoprobacteraceae	[114, 115]	0.00	5.20	5.77	3.21	0.41	0.05
f_Caloramatoraceae	[116, 117]	0.00	11.38	0.94	1.11	2.19	0.04
f_Comamonadaceae	[118]	0.55	0.01	0.00	0.00	0.02	0.27
f_Exiguobacteraceae	[119-122]	0.00	0.02	0.01	5.37	0.01	0.01
f_ Hungateiclostridiaceae	[123, 124]	1.07	0.00	0.00	0.00	0.00	0.00

		Mud	TYC	TYC	TYC	TYC	Ace
f_ Peptostreptococcaceae	[125]	0.02	0.00	0.01	1.06	0.00	0.04
f_SBR1031 (c_ Anaerolineae)	[126]	0.11	0.00	0.00	0.00	0.00	0.00
f_SCADC1-2-3	[25, 29]	2.08	0.03	0.03	0.03	0.03	0.02
f_Thermacetogeniaceae	[19]	0.00	0.02	2.35	2.20	3.78	0.02
f_Thermaerobacteraceae	[72, 73]	0.33	0.01	0.00	0.00	0.01	0.07
f Thermoactinomycetaceae	[127]	1.23	0.04	0.02	0.04	0.04	0.03
f_Weeksellaceae	[128, 129]	0.44	0.00	0.00	0.00	0.05	0.22
f_ Xanthomonadales	[130]	0.00	0.01	0.00	0.00	0.03	0.17
H2-generating organotrophs		3.51	23.73	22.70	22.89	20.91	5.69
f_Desulfotomaculales	[56-58]	0.05	0.02	1.89	0.45	0.05	0.00
f_Fervidobacteriaceae	[131, 132]	2.50	21.81	8.14	7.15	1.17	5.08
f_Pirellulaceae	[133]	0.86	0.00	0.82	0.56	0.11	0.00
f_Thermotogaceae	[31, 55, 61, 62, 134]	0.10	1.89	11.85	14.74	19.58	0.60
S0, S2O32-, SO42–reducing organotrophs		17.20	49.61	24.98	26.94	12.77	67.96
f_Archaeoglobaceae	[74]	0.00	0.01	0.76	0.41	0.41	0.02
f_Caldisericaceae	[135]	0.00	1.18	0.00	0.00	0.00	0.03
f_Desulforudaceae	[136, 137]	0.25	0.00	0.00	0.00	0.03	0.00
f Hydrothermae (candidatus)	[59, 60]	0.06	0.02	0.02	0.02	0.01	0.02
f_ Synergistaceae	[138, 139]	0.00	0.00	0.00	0.00	0.14	0.00
f_Thermodesulforhabdaceae	[27, 76]	1.37	0.92	1.06	0.86	0.39	46.77
f_Thermaceae	[77, 78]	7.44	0.06	0.04	0.05	0.05	0.85
f_Thermoanaerobacteraceae*	[69-71]	0.01	5.39	7.49	4.85	1.31	0.13
f_Thermococcaceae	[64, 65]	0.02	24.78	7.28	3.13	9.23	19.51
f_Thermodesulfovibrionaceae	[29, 75]	8.05	0.08	0.04	0.04	0.07	0.48
f_Thermovenabulales	[140-142]	0.01	17.17	8.29	17.59	1.13	0.14
OTHER		8.65	1.56	5.05	8.41	18.85	3.41

* Colored based on relative abundance. Blue low abundance, red high abundance.

** Includes the Incertae Sedis Gelria





Supplementary Figure 1

Supplemental material, figure 1 GCMS chromatograms showing general hydrocarbon degradation for crude oil containing cultures and sterile controls. A) S^0 and crude oil enrichment culture compared to sterile control. B) Ferrihydrite and crude oil enrichment culture compared to sterile control C) crude oil containing enrichment culture with no added electron acceptor

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