Sulfur-Cycling in Methane-Rich Ecosystems: 
Uncovering Microbial Processes and Novel 
Niches

Thesis by 
Abigail Green Saxena

In Partial Fulfillment of the Requirements for the degree 
of 
Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY 
Pasadena, California 
2013
(Defended 20 May 2013)
Chapter 1

*This chapter, written by Abigail Green Saxena, was first published in Environmental Microbiology:
Active sulfur cycling by diverse mesophilic and thermophilic microorganisms in terrestrial mud volcanoes of Azerbaijan

A. Green-Saxena², A. Feyzullayev³, C.R.J. Hubert⁴, J. Kallmeyer⁵, M. Krueger⁶, P. Sauer⁵, H.-M. Schulz⁷ and V.J. Orphan¹

Divisions of ¹Geological and Planetary Sciences and ²Biology, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125
³Petroleum Geology and Geochemistry Department, Geology Institute of ANAS, H. Cavid avenue 29a, Baku, AZ1143, Azerbaijan
⁴School of Civil Engineering & Geosciences, Devonshire Building, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom
⁵Institute of Earth and Environmental Sciences, University of Potsdam, Haus 27, Zi. 0.34, Karl-Liebknecht-Str. 24 14476 Golm, Germany
⁶Fields of Geomicrobiology and Resource Geochemistry, Bundesanstalt fuer Geowissenschaften und Rohstoffe (BGR; Federal Institute for Geosciences and Natural Resources), Stilleweg 2, D-30655 Hannover, Germany
⁷GeoForschungsZentrum Potsdam, Section 4.3, Telegrafenberg, B 424, D-14473 Potsdam, Germany
SUMMARY

Terrestrial mud volcanoes (TMVs) represent geochemically diverse habitats with varying sulfur sources and yet sulfur cycling in these environments remains largely unexplored. Here we characterized the sulfur-metabolizing microorganisms and activity in 4 TMVs in Azerbaijan. A combination of geochemical analyses, biological rate measurements and molecular diversity surveys (targeting metabolic genes aprA and dsrA and SSU ribosomal RNA) supported the presence of active sulfur-oxidizing and sulfate-reducing guilds in all 4 TMVs across a range of physiochemical conditions, with diversity of these guilds being unique to each TMV. The TMVs varied in potential sulfate-reduction rates (SRR) by up to 4 orders of magnitude with highest SRR observed in sediments where in situ sulfate concentrations were highest. Maximum temperatures at which SRR were measured was 60°C in two TMVs. Corresponding with these trends in SRR, members of the potentially thermophilic, spore-forming, Desulfotomaculum were detected in these TMVs by targeted 16S rRNA analysis. Additional sulfate-reducing bacterial lineages included members of the Desulfbacteraceae and Desulfobulbaceae detected by aprA and dsrA analyses and likely contributing to the mesophilic SRR measured. Phylotypes affiliated with sulfide-oxidizing Gamma- and Betaproteobacteria were abundant in aprA libraries from low sulfate TMVs, while the highest sulfate TMV harbored 16S rRNA phylotypes associated with sulfur-oxidizing Epsilonproteobacteria. Altogether, the biogeochemical and microbiological data indicate these unique terrestrial habitats support diverse active sulfur-cycling microorganisms reflecting the in situ geochemical environment.
INTRODUCTION

Terrestrial mud volcanoes (TMVs) occur where high fluid pressure in the deep subsurface results in the transport of mud, water and gas to the surface (Feyzullalev and Movsumova, 2010; Niemann and Boetius, 2010). This process creates diverse morphological features rich in methane and other hydrocarbons (Dimitrov et al., 2002), and hosting a suite of electron acceptors including oxygen, nitrate, iron, manganese and sulfate (Planke et al., 2003; Mazzini et al., 2009; Alain et al., 2006; Chang et al., 2011). Mud volcanoes are a major source of methane flux to the atmosphere (6-9 Tg/year; Etiope and Milikov, 2004), and geochemical and microbiological studies thus far have primarily focused on microorganisms and processes involved in the oxidation of methane, including the detection of anaerobic methane-oxidizing archaea (ANME) in individual volcanoes (Niemann et al., 2006; Alain et al., 2006; Schulze-Makuch et al., 2011; Wrede et al., 2011; Yang et al., 2011; Chang et al., 2011). 16S rRNA surveys of TMVs in Romania, Taiwan and China contain a large number of putative sulfate-reducing Deltaproteobacterial phylotypes, consistent with the potential for sulfate-dependent anaerobic methane oxidation (Alain et al., 2006; Chang et al., 2011; Yang et al., 2011), however in some cases sulfate reduction rates (SRR) appear to be significantly greater than anaerobic methane oxidation rates (Alain et al., 2006).

The ecology of organisms involved in sulfur cycling remains largely unexplored in TMVs. Unlike marine MVs, very little is known about chemosynthetic communities such as sulfur-oxidizing bacterial (SOB) populations in TMVs (Niemann and Boetius, 2010) and
while sulfate-reducing bacterial (SRB) phylotypes based on 16S rRNA have been reported from individual TMVs, their diversity in these habitats has not been examined by analyzing sulfur-cycling functional genes. TMVs have the potential for supporting active sulfur cycling with typical sulfate concentrations of approximately 2 mM (Planke et al., 2003; Mazzini et al., 2009; Alain et al., 2006; Nakada et al., 2011; Yakimov et al., 2002), and these natural venting structures may also serve as a window into sulfur-cycling processes in the deep biosphere. While previous microbiological studies have characterized individual TMVs, regions hosting multiple TMVs also make it possible to focus on the same microbial guilds and processes across several sites.

Azerbaijan and its offshore expanses in the Caspian Sea represent one of the most densely populated regions of mud volcanoes and mud volcanism is one of the major factors controlling oil and gas fields in the region. Unusually high $\delta^{13}$C values of CO$_2$ and bicarbonates in the TMVs are thought to result from biodegradation of oil (Feyzullayev and Movsumova, 2010), however very little is known about the microbial communities in these volcanoes. Multiple studies have characterized the complex plumbing system of the TMVs that are capable of transporting mud and fluids from origins as deep as 10 km or greater (Mazzini at al., 2008; Planke et al., 2003, and references therein), and records of eruption history as well as geochemical and isotopic compositions of emitted oil, gas, mud and water exist for several prominent TMVs in the region (Etiope et al., 2004; Feyzullayev and Movsumova, 2010; Guliyev et al., 2001; Mazzini et al., 2009; Planke et al., 2003).

Analyses of oil source-rocks ejected from mud volcanoes of Azerbaijan revealed low organic sulfur contents (less than 0.03% of the organic matter) with the majority of sulfur occurring as pyrite (Isaksen et al., 2007). Sulfate concentrations appear to vary widely with studies
reporting concentrations that are on average ~ 2 mM, but range of values from ~ 10 µM to > 30 mM (Planke et al., 2003; Mazzini et al., 2008). This diverse system of mud volcanoes provides a set of distinct habitats in which to study natural variations in the \textit{in situ} interplay of sulfur-cycling communities and their geochemical environments. Here we conducted a comparative geomicrobiological study of 4 discrete TMVs in Azerbaijan (D: Dashgil, B: Bakhar, P: Perekushkul and BJ: Boransyz-Julga) sourced from deep-seated fluids in order to: 1) characterize sulfur-cycling microbial communities in these unique environments, 2) place these communities in a meaningful context via geochemical and microbial rate measurement analyses and 3) examine the potential transport of thermophilic sulfur-cycling microorganisms from the deep subsurface.

**RESULTS**

**Geochemical characterization**

Inorganic and organic geochemistry of pore fluids from 4 mud volcanoes were analyzed with a particular emphasis on sulfur and carbon species (Tables 1, S4). In general, values were similar to previous reports of geochemistry from TMVs in this area (Planke et al., 2003; Mazzini et al., 2009). Sulfate contents ranged between 0.47 and 1.64 mM in D sediments except for in sample D3, which was below detection (< 0.16 mM). P surface (P1S) and deep (P1D) samples contained 0.31 and 0.80 mM sulfate, respectively. B and BJ had sulfate levels below the detection limit (< 0.16 mM). The sulfate concentrations ranged from less than 0.16 to 1.64 mM and are within the range of previously reported values from TMVs in Azerbaijan (Planke et al., 2003; Mazzini et al.,
2009) as well as those reported from TMVs in Romania (2 mM; Alaine et al., 2006), Italy (0.07 mM, Wrede et al., 2011) and China (14 mM; Yang et al., 2011). While values vary widely, none of them approach that of seawater in general (approximately 28 mM), or the Caspian Sea in particular (approximately 33 mM; Planke at al., 2003 and references therein).

Higher Cl values of D salse lakes/pools (312 to 464 mM) versus gryphons from D, P and BJ (56 to 68 mM) are in agreement with previously reported trends from this region, and may reflect a deep water source resulting from the dehydration of clays in the gryphons, while the salse lakes and pools may be fed by more shallow meteoric water source with higher solutes resulting from in situ evaporation (Mazzini et al., 2009). Higher Cl/Br ratios in salse lakes (relative to gryphons) in this region have been previously reported and may be derived from the dissolution of halite crusts which form on the outside flanks of gryphons occurring at higher elevations than salse lakes (Mazzini et al., 2009).

Reduced sulfur species associated with the solid phase materials were divided into three phases named for the compounds used to liberate them: AVS (acid-volatile sulfur; hydrogen sulfide and monosulfides): CRS (chromium-reducible sulfur, mainly pyrite): DMF (dimethylformamide-soluble fraction, mainly elemental sulfur). Values from all samples analyzed were similar, with disulfides comprising > 99% of all reduced sulfur except BJ, which contained relatively lower amounts of disulfides (96% from sample S1; 72% from S2) the difference being made up by monosulfides (Tables 1, S4).

Microbial Microcosm Measurements
Biogenic CO$_2$ production under aerobic conditions (heterotrophic respiration) was at least three orders of magnitude greater than CO$_2$ production under anaerobic conditions. In addition, anaerobic sulfide production (in the presence and absence of methane), indicative of active sulfate reduction, was also two orders greater than anaerobic CO$_2$ production (Table 2). Sulfide and anaerobic CO$_2$ production rates ranged from $1.4 \times 10^4$ to $6.7 \times 10^4$ and $2.7 \times 10^2$ to $6.3 \times 10^2$ (nmol cm$^{-3}$ day$^{-1}$), respectively. Higher rates of sulfide and anaerobic CO$_2$ production were measured from D samples suggesting potentially higher in situ sulfate reduction rates in the D salse lakes (D1 and D3).

**Potential sulfate-reduction rates**

Sulfate reduction rates (SRR) were measured from three of the four mud volcanoes; D (deep pool sample, D2D), B (gryphon sample B1) and BJ (gryphon sample BJ1). Sediment slurries were incubated for 8 hours with sulfate radiotracer at temperatures ranging from approximately 10°C to 82°C, under three different experimental incubation conditions: following 24 hour pre-incubation, following 48 hour pre-incubation, and following 48 hour pre-incubation with VFAs. Results were highly variable with SRR spanning four orders of magnitude between sites and depending on incubation temperature, pre-incubation time, and VFA addition. Incubations with sediment from BJ, located inland from D and B (Figure 1), resulted in a temperature profile for SRR with a narrow temperature optimum around 30°C and no detectable thermophilic activity (Figure 2c). Results from D and B varied significantly in response to VFA stimulation and revealed different temperature optima for sulfate reduction despite both D and B occurring in the same hydraulic system and rock formation. Sulfate reduction maxima in the thermophilic...
range were measured for B and D sediments at 54°C and 60°C, respectively, with SRR below detection at higher temperatures up to 82°C.

D samples pre-incubated for 24 and 48 hours resulted in similar temperature-activity profiles, with the highest SRR measured at 32.5°C. While sulfate reduction was not detected above 40°C after 32 hours (i.e., 24 hour pre-incubation followed by 8 hours with radiotracer), SRR obtained for 48-56 h revealed a second peak in SRR between 50 and 70°C. This response was dramatically amplified by VFA amendment, which resulted in SRR up to 250-fold higher at these high temperatures than in the unamended samples (Figure 2a), similar to previous reports of VFA supporting high SRR in cold marine sediments incubated at these temperatures (Hubert et al. 2010). In B sediments, SRR (ranging from 0.78-3.70 nmol/cm$^3$/d) were much lower than those measured in D sediments (2.38-2359.88 nmol/cm$^3$/d) and, as observed for D, the temperature optima depended on both the pre-incubation time and the addition of VFAs. For the two unamended incubations, the temperature range for sulfate reduction was broad and showed several rate maxima between 15°C and 40°C. Notably, SRR determined after the 24- and 48 hour pre-incubations followed slightly different temperature-activity profiles, with rates measured after 24 hours being 1.5-fold higher between 25 and 32°C than after the 48 hour treatment. VFA amendment to B samples stimulated SRR that were slightly higher than in unamended samples, with two distinct peaks at 30°C and 54°C and a third minor peak around 15°C (Figure 2b). The potential sulfate reduction rates were also low in samples from BJ, with one narrow peak at 30°C determined for all three incubation treatments. SRR in samples pre-incubated for 48 hours (3.37 nmol/cm$^3$/d) were approximately threefold
greater than in the 24 hour sample (1.00 nmol/cm³/d). Unlike the samples from D and B, VFA addition to BJ samples did not result in higher SRR (2.34 nmol/cm³/d; Figure 2c).

**Determination of microbial abundance**

Quantitative 16S rRNA gene analyses from D and B samples collected in 2007 suggest bacteria are an order of magnitude more abundant than archaea in D salse lakes (D1 and D3), while the two Domains appear equally abundant in the small salse lake sample from B (Table S3). The depth from which the sample was taken did not appear to influence these trends. Across all samples, bacterial 16S rRNA gene copies range from approximately $1 \times 10^7$ to $1 \times 10^9$ copies/g, and archaeal 16S rRNA gene copies range from $1 \times 10^7$ to $1 \times 10^8$ copies/g.

**Molecular characterization of sulfur-cycling bacteria**

Samples for phylogenetic analysis were selected based on sulfate-reduction rate data and *in situ* sulfate concentrations. Here we targeted the *aprA* gene (adenosine-5-phosphosulfate [APS] reductase) encoding the enzyme required for dissimilatory sulfate-reduction and used in many sulfur-oxidation pathways (Meyer and Kuever, 2007a). Two *aprA* clone libraries were constructed from samples of the same Dashgil pool; one from the surface (D2S) and one collected approximately 4 meters below the surface (D2D). Three clone libraries were constructed from gryphon samples. The B (B1) and BJ (BJ1) samples were collected at the surface of the gryphon, and the P (P1D) sample was recovered from approximately 2 meters below the surface.
The *aprA* gene sequences recovered from 4 of the 5 mud volcano samples contained representatives from several groups within the Gamma, Beta, and Deltaproteobacteria representing multiple sulfate-reducing and sulfur-oxidizing bacterial clades. The exception to this was BJ, where 96% of the recovered *aprA* sequences were affiliated with sulfide-oxidizing Betaproteobacterial members related to *Thiobacillus* (Figure 3a). Gammaproteobacterial clones from the *aprA* lineages I and II (as defined in Meyer and Kuever; 2007b) were present in all but the BJ sample, with lineage I representing the majority of these clones. Gammaproteobacterial *aprA* lineage I sequences from B, P and both D samples formed two distinct clusters each of which grouped with distinct *aprA* sequences from two *Thioalkalivibrio* strains (Figure 3a). Sequences within lineage I also included representatives grouping within the Chromatiaceae (D2S) and environmental sequences reported from the ground waters of an evaporative, calcareous, salt lake. Putative sulfide-oxidizing Gammaproteobacterial *aprA* lineage II clones from B, P and both D samples also formed two distinct clusters, one affiliated with environmental clones from sulfate-reducing bioreactors treating mine drainage (Hiibel et al., 2008); the other grouping with members of *Thiodiciton* sp., within the Chromatiaceae. Only one sequence, retrieved from BJ, appears loosely affiliated with an uncultured Alphaproteobacteria (Figure 3a).

All sulfate-reducing bacterial sequences from *aprA* libraries grouped with mesophilic Deltaproteobacteria, with the exception of a clone recovered from D2D, which grouped within the gram-posistive *Desulfotomaculum* subcluster 1b, which includes thermophilic sulfate reducers (Meyer and Kuever, 2007c). The majority of sequences within the Desulfo bacteraceae were from D surface and deep samples (D2D and D2S), several of
which formed a distinct cluster distantly associated with *Desulfoarcina variabilis*. Clones from P, B and D surface samples made up the majority of Desulfobulbaceae sequences, mainly grouping into 3 clusters with either no described relative or a distant association with *Desulfurivibrio alkaliphilus* (Figure 3b).

The ratio of *aprA* clones recovered from sulfur-oxidizing versus sulfate-reducing bacteria (SOB:SRB) varied between samples, with SOB clones dominant in BJ (98% SOB) and B (68% SOB). The SOB populations in these samples were distinct, B contained *aprA* clones within the Gammaproteobacteria (43% *aprA* lineage I, 25% lineage II) while BJ contained 96% Betaproteobacterial (*aprA* lineage II) clones and a single clone putatively from the Alphaproteobacteria. B also contained clones (30% of library) from the Deltaproteobacterial family Desulfobulbaceae while BJ did not contain SRB-affiliated *aprA* sequences.

P and D surface and deep samples were dominated by *aprA* clones affiliated with SRB lineages. The D deep sample (D2D) was almost entirely dominated by Desulfobacteraceae (83%), with only a single clone each from Desulfobulbaceae and Desulfotomaculum (Figure 3b). Similar to D2D, the P sample was collected several meters below the gryphon surface, but had an SRB profile more similar to the D2S surface sample. The majority (56%) of P clones grouped within the Desulfobulbaceae with 4% affiliated with Desulfobacteraceae. The D surface sample also contained a number of *aprA* sequences from the Desulfobulbaceae and Desulfobacteraceae family (63% and 18%, respectively). Additionally, there appeared to be a trend between libraries dominated by SRB sequences and the occurrence of Gammaproteobacterial lineage I clones comprising the dominant SOB group.
As an independent check of the aprA results, a single dsrA library was constructed from D2D. Similar to the aprA library from the same sample, the recovered dsrA sequences were dominated (84%) by Desulfobacteraceae, with one Desulfobulbaceae clone and 9 clones with no closely described relatives (Table S2). A phylogenetic analysis using known reference sequences placed several of these unidentified clones within a cluster of Desulfotomaculum sequences (data not shown).

Molecular characterization of 16S rRNA bacterial diversity

A bacterial 16S rRNA survey was completed from the D2D pool sample. Of the recovered diversity, 59% of the clones were Epsilonproteobacteria, followed by Chloroflexi (19%), Deltaproteobacteria (9%), and a low number of sequences associated with the Firmicutes (5%) and Bacteroidetes (3%). Epsilonproteobacterial sequences were highly similar (96-99% max identity) to environmental clones from the facultatively anaerobic SOB Sulfurovum sp. and all Chloroflexi clones were highly similar (96%) to environmental clones from landfill leachate pond sediments (Liu et al., 2011). Among the Deltaproteobacteria, clones were similar to environmental sequences from wetlands (3 clones, 94% max identity) and a river (2 clones, 99%), as well as strains isolated from oil reservoirs (2 clones, 95%; Table S2). Firmicutes and Bacteroidetes clones were similar to environmental sequences from a soda lake (4 clones, 93%) and river (2 clones, 98%) respectively. To further assess the occurrence and distribution of putative thermophilic SRB contributing to the observed 60°C SRR, we used 16S rRNA primers targeting the genus Desulfotomaculum (Stubner and Meuser, 2000), since this genus was detected in both dsrA and aprA gene libraries. This 16S rRNA gene-based approach independently
confirmed the presence of *Desulfotomaculum* sequences in Dd and B samples.

**DISCUSSION**

Here we characterize the geochemistry and sulfur-cycling communities of four terrestrial mud volcanoes in Azerbaijan: D (Dashgil) and B (Bakhar), located near the Caspian Sea, and two inland TMVs, P (Perekyushkul) and BJ (Boransyz-Julga), located on the foothills of the Great Caucasses (Figure 1). Molecular and geochemical data support the presence of active sulfur-oxidizing and sulfate-reducing guilds across a range of physiochemical conditions. While both guilds were present in all four TMVs, the diversity within each guild was unique for each mud volcano suggesting a complex interplay of ecological and environmental factors influence the structure of sulfur-metabolizing communities. Molecular data revealed SOB were present in mud volcanoes with low and high sulfate levels and co-occurred with an active SRB population. *AprA* phylotypes affiliated with SOB revealed putative chemosynthetic metabolisms including sequences clustering with obligate aerobes and facultative anaerobes collectively capable of coupling the oxidation of a variety of sulfur species to the respiration of both oxygen and nitrate. SRB community analyses together with SRR measurements support a predominance of *Desulfobulbaceae* members and active sulfate reduction across a range of temperatures with potential rates reflecting ambient sulfate levels. Thermophilic sulfate reduction was also detected in these approximately 20°C mud volcanoes and together with molecular detection of putatively thermophilic *Desulfotomaculum* spp., supports the hypothesis that mud volcanoes transporter thermophilic microorganisms from deep warm habitats up to the cooler surface
where they have been previously discovered as endospores (Hubert et al., 2009).

**Microbial Ecology of the Azerbaijan Mud Volcanoes**

The investigated mud volcanoes support microbial assemblages similar in abundance to other TMVs with bacterial abundance being greater than or equal to that of archaea (Table S3), depending on site and depth examined (Alain et al., 2006; Chang et al., 2011; Schulze-Makuch et al., 2011). Molecular analyses and rate measurements presented here indicate that bacterial diversity is moderate and dominated by meso- and thermophilic sulfur-cycling microorganisms.

Geochemical data suggest the large salse lakes and smaller pools, both of which continuously emit gas and water but very little sediment, have a shallower fluid source than gryphons (conical-shaped mounds emitting viscous mud, gas and water). These data are similar to prior studies from this region (Etioppe et al., 2004; Mazzini et al., 2009), and may also explain differences observed in sulfate-reducing bacterial phylotypes retrieved from these distinct habitats. *In situ* sulfate concentrations were highly variable and did not appear to be correlated with specific habitats (salse vs. gryphon) or proximity to the Caspian Sea, and may therefore result from deep sources. Measurements of abundance and composition of reduced sulfur species (Tables 1, S4) are consistent with published studies from this region, revealing sulfur to be primarily in the form of disulfides such as pyrite (Isaksen et al., 2007). Monosulfides and elemental sulfur may therefore have a short residence time in these systems due to rapid sulfur cycling. Rock-Eval pyrolysis revealed higher concentrations of free hydrocarbons in sulfate-depleted volcanoes (B and BJ) suggesting
more labile carbon may be available to microbes in these sites, perhaps resulting from a scarcity of heterotrophic SRB.

Relevant microbial processes affecting carbon cycling that were not directly measured here include methanogenesis and methanotrophy. Studies of TMVs have reported evidence of anaerobic methane-oxidizing ANME archaea by CARD-FISH, molecular, and rate analyses (Alain et al., 2006; Schulze-Makuch et al., 2011; Wrede et al., 2011; Yang et al., 2011; Chang et al., 2011). In the present study, CARD-FISH analyses also revealed the occurrence of ANME/SRB aggregates (Figure S2), and sulfide production was observed in anaerobic microcosms incubated with methane (Table 2).

**Sulfur Cycling**

Microbial rate measurements and molecular analyses were used to further examine sulfur cycling in these dynamic environments. Unlike marine MVs, very little is known about chemosynthetic communities such as sulfur-oxidizing bacterial (SOB) populations in TMVs (Niemann and Boetius, 2010), though these communities can be a significant source of primary production (Levin et al., 2002). *AprA* phylotypes associated with putative SOB were recovered from all samples and were similar in three out of the four TMVs. Phylogenetic analyses reveal that phylotypes from B, D and P cluster together to the exclusion of reference sequences from cultured species (Figure 3a). The majority of these sequences have as their closest relative *Thioalkalivibrio* spp., an aerobic Gammaproteobacterial genus isolated from soda lakes (Sorokin 2001). The occurrence of these potential alkalophilic SOB phylotypes in P is also consistent with the recovery of SRB phylotypes from the same clone library (48/93 total clones), which fell within a
cluster of sequences most closely related to *Desulfurivibrio alkaliphilus* (Figure 3b).

The pH values of all investigated TMVs were approximately 8.0; with waters of P (and BJ) with a total mineralization in the range of 1.6 to 2.6 g/l, defined as highly alkaline, hydrocarbonate-sodium type water compared to D (and B) which show total mineralization in the range of 2.5 to 8.2 g/l (data not shown). BJ, while sharing some physical and geochemical features with other TMVs, had distinct *aprA* phylotypes, most of which (96%) grouped closely together in one cluster of Betaproteobacteria including sequences from *Thiobacillus denitrificans*, a facultative anaerobe capable of respiring nitrate and oxygen. 16S rDNA phylotypes from D also provided evidence for the presence of SOB with Epsilonproteobacteria comprising a significant proportion of recovered sequences. As both putative aerobic and nitrate-respiring SOB phylotypes were recovered, these data suggest the sulfur-oxidizing guild may be common to TMVs in the region with the environment dictating the dominant SOB lineage (Table S2). Total nitrogen and $\delta^{15}$N were measured with similar values across all TMVs. Although the composition of nitrogen species was not measured here nitrate versus oxygen availability could be a driver of dominant SOB lineages. Future studies focusing on nitrogen cycling in TMVs may reveal SOB as an important microbial component.

SRB communities can be broadly categorized into two groups based on their carbon oxidation pathways; complete oxidizers are capable of complete mineralization of organic substrates to CO$_2$, while incomplete oxidizers excrete acetate as a final byproduct (Canfield, 2005). There exists a taxonomic relationship between carbon oxidation pathway and many genera or even families of SRB; most members of the *Desulfobacteraceae* family are complete oxidizers while most members of the *Desulfobulbaceae* are not (Kuever at al., 2005).
CARD-FISH analyses revealed the occurrence of *Desulfobacteraceae* (Figure S2) and Desulfobulbaceae (data not shown) cells. Of the SRB phylotypes retrieved from the investigated mud volcanoes, putative incomplete carbon-oxidizing genera within the *Desulfobulbaceae* family were dominant at all sites except the deep Dd sample, which is dominated by *Desulfobacteraceae* (Figure 3b). All clone libraries originated from gryphon mud volcanoes, except for the D libraries, which were associated with a mud volcano pool. While gryphons contain viscous mud that may be more susceptible to mixing by rising gas bubbles throughout the depth column, pools contain water with comparatively minor amounts of fine sediment overlying a more discrete benthic layer. Interestingly, the diversity recovered from D, sampled at the surface (Ds) and at the bottom sediment (Dd) showed a dominance of complete carbon oxidizers in the underlying sediment layer but not in the surface sample. *DsrA* gene surveys from deep Dd also confirmed a significant fraction of recovered sequences were associated with complete carbon-oxidizing *Desulfobacteraceae* (Table S2). The P gryphon was also sampled at depth (2 m) however here showed a dominance of putative incomplete carbon-oxidizing Desulfobulbaceae. Cultured representative of incomplete oxidizing SRB are known to have comparatively faster growth rates, and outcompete complete oxidizing SRB in enrichments with substrates like lactate and thus may have a selective advantage in habitats where organic matter input is variable (Canfield, 2005). It is possible that gryphons and the shallow surfaces of salse lake waters are more dynamic environments giving incomplete carbon-oxidizers an advantage.

Microbial rate measurements were performed in order to assay carbon utilization and
temperature optimum among active SRB communities. Previous studies have confirmed an approximate 2:1 stoichiometry of CO$_2$ produced per sulfate consumed in habitats where sulfate reduction represents the primary means of carbon mineralization (Thamdrup and Canfield, 1996; Vandieken et al., 2006). In the present study CO$_2$ and sulfide production rates based on microcosm experiments revealed anaerobic CO$_2$ production was several orders of magnitude less than sulfate consumption (Table 2), consistent with a significant fraction of sulfate reduction carried out by incomplete carbon-oxidizing SRB. In most TMVs sampled, the addition of volatile fatty acids (VFAs) did not substantially stimulate SRRs suggesting these samples were not limited by bioavailable carbon and consistent with Rock Eval analyses (Figure 2). The exception was the deep Dd sample in which VFA addition caused a several orders of magnitude increase in SRR. Notably, aprA phylotypes recovered from this sample were uniquely related to putative autotrophic genera suggesting a potential adaptation to carbon limitation.

**Evidence of Deep Biosphere Activity**

Terrestrial mud volcanoes have been proposed to serve as conduits that connect Earth’s surface environments with the underlying deep biosphere whereby the same processes that trigger mud volcanism also lead to a transport of materials from great depths (Hubert et al., 2009; Niemann and Boetius, 2010). While ambient surface mud temperatures of approximately 20 °C were measured in the current study (Table 1), conduits of > 10 km have been modeled from Azeri mud volcanoes (Planke et al., 2003) suggesting a potential for the transport of organisms from deeper warmer underlying strata. Maximum SRR in the thermophilic range were observed from 20 °C mud collected at B and D, exhibiting
temperature optima (55 °C and 60°C, respectively) within the activity range of thermophilic SRB belonging to the genus *Desulfotomaculum*. SRR at these high temperatures were stimulated by the addition of VFAs, which members of this genus are known to use as electron donors (Widdel, 2006). Targeted 16S rRNA primers specific for the genus *Desulfotomaculum*, which was detected in both *dsrA* and *aprA* gene libraries, independently confirmed the presence of *Desulfotomaculum* sequences in those samples exhibiting sulfate reduction at high temperatures.

*Desulfotomaculum* have been detected in several deep subsurface habitats such as 5 km deep faults and 3.2 km gold mine boreholes (Baker et al., 2003; Moser et al., 2005) and mud volcanoes were hypothesized as a mechanism of transport of thermophilic SRB of the genus *Desulfotomaculum* from deep warm habitats to the arctic seabed (Hubert et al., 2009). This idea is strongly supported by our observation of high SRR in mud volcano samples originally at 20°C *in situ* that are heated to 50-70°C, and by the detection of putatively thermophilic *Desulfotomaculum* spp. in the same samples. Based on our *in situ* temperature measurements (Tables 1, S4), and the reported geothermal gradient in the South Caspian Basin (Planke et al., 2003; and references therein) temperatures of 50 to 65°C (optimal SRR as shown in figure 2 and consistent with the thermal range of several *Desulfotomaculum*; Widdel, 2006) exist at approximately 2 to 3 km below the surface. Consistent with a lack of SR detection above 60°C, we saw no molecular evidence of hyperthermophilic sulfate reducers, either archaea or bacteria.
CONCLUSIONS

Mud volcanism in Azerbaijan is one of the controlling factors for the vast oil and gas fields in this area; thus elucidating microbial processes in this region is important as these processes can play an important role in the degradation of hydrocarbon inside the reservoirs. The ability to analyse the geochemistry and microbial diversity and processes of distinct TMVs afforded by their high density in this region gives new insights into the interplay between S-cycling microorganisms and their environment. Sulfur cycling in TMVs has been largely unexplored and here we provide a comparative view of the microbial communities and processes involved in sulfur cycling in multiple TMVs of Azerbaijan. Sulfate-reducing and sulfur-oxidizing bacterial guilds were present in all TMVs but differed at the genus level between individual mud volcanoes. Detection of thermophilic SRB in 20°C habitats suggest that these TMVs, with conduits extending 10 km or more into deep thermic sediment, may actively transport microorganisms adapted to the deep biosphere.

EXPERIMENTAL PROCEDURES

Site Descriptions

Four mud volcanoes in the South Caspian Basin of eastern Azerbaijan were investigated: Dashgil (D), Bakhar (B), Perekyushkul (P) and Boransyz-Julga (BJ). Samples were taken from the following TMV features (described in Mazzini et al., 2009): gryphons (conical-shaped features less than 3 m in height which continuously emit gas, water, oil and viscous mud from their craters), pools (small round features with a diameter
up to 2 m, which continuously release water and gas with a minor amount of fine sediment) and salse lakes (lake-like features up to 30 m in diameter and 10 m deep, which vigorously vent large quantities of gas and water with only a limited amount of mud).

Despite regional and geological differences there is evidence that all four investigated mud volcanoes are sourced from the Maikop Series (Berner et al., 2009), which is Oligocene-Low Miocene in age and rich in organic carbon. Sediments of the Maikop Series are unconsolidated due to rapid burial and overpressure. Overpressuring is enhanced by biogenic and thermogenic gas generation, and results in upward migration at tectonically weak zones or due to earthquakes. Although all four mud volcanoes likely share this common source, D and B are located near the Caspian seaside lying on young Quaternary sediments while BJ and P are situated on the SE foothill of Great Caucasus and lie on comparatively older Oligocene-Miocene deposits (Figure 1).

D was photographed in 1997 by Hovland and colleagues, and together with B and P can be found in published maps of the South Caspian Basin along with background geochemical data (Etiöpe et al., 2004; Feyzullayev and Movsumova, 2010; Guliyev at al., 2001; Planke et al., 2003; Mazzini et al., 2009). D has more than 60 gryphons and salses, and has erupted at least 6 times since 1882; B has around 30 gryphons and salses, has erupted 11 times since 1853, the last recorded eruption occurred in 1992 (Etiöpe et al., 2004 and references therein). B is considered to have higher eruptive potential (Etiöpe et al., 2004), but both D and B have high seep activity (Planke et al., 2003). To our knowledge, there are no prior studies of BJ. Both P and BJ are visible from public satellite imaging (see Tables 1, S4 for GPS coordinates) and appeared to be dominated by uplifted clusters of gryphons during the October 2008 trip (Figure S1). Several of the gryphons in P
and BJ had white crust on the dry outer flanks. In BJ, dead arthropods (mainly beetles) were observed floating on the surface along the sides of several gryphons, along with a visually identified microbial mat. While mud volcanoes are considered dormant in the interval between eruptions (Mazzini et al., 2009), all four TMVs investigated exhibited active seepage of gas and mud.

**Sampling**

Samples were collected in 2007 from two D salse lakes (D1 and D3 in 2008 data) and one small salse lake within a satellite vent of B (see Planke et al., 2003 and Mazzini et al., 2009 for location of satellite relative to main vent). During a second collection trip in October 2008 samples were collected from D, the same B satellite vent, BJ, and P. Unless stated otherwise, all samples were collected directly into sterile 50 ml falcon tubes. Due to the morphological diversity of D and abundance of published background data, multiple features were sampled within this volcano. Sample D1 was taken from a large, actively bubbling salse lake (referred to as “Salse A” in Planke et al., 2003 and Mazzini et al., 2009). These samples were taken from the surface of the lake approximately 1 m from the shore using an extendable pole. Ds and Dd were taken from the surface and depths, respectively, of a small pool approximately 3 m from D1 (see Figure S1 for photograph). Deeper sample Dd was collected from approximately 4 m below the surface of the salse using a 65 cm drop core. Mud for microbial analyses was sampled from the center portion of the core. D3 samples were taken from a smaller salse lake (“Salse B” in Planke et al., 2003 and Mazzini et al., 2009) with visibly clearer water than that of D1. Samples were scooped from the bottom of this salse using a large ladle attached to the end of an...
extendable pole. D4 samples were collected directly into a falcon tube from the thick bubbling surface of a gryphon. B samples were also taken directly from the surface of an actively bubbling gryphon at a site known as the Bakhar satellite vent. At the BJ site, surface material from a viscous gryphon was sampled (BJ) along with sediments from the bottom of a less viscous gryphon (BJ2). Ps and P were taken from the surface and depths, respectively, of a gryphon in P. Deeper sample P was taken from the bottom of a 65 cm drop core extended approximately 2 m below the surface.

Field conditions required that all samples be kept at ambient temperature until the end of each sampling day (approximately 5 hours) when they were processed and stored at the Geology Institute of ANAS (The Azerbaijan National Academy of Sciences) research lab in Baku prior to their shipment to either GFZ, Potsdam, Germany or CIT, USA.

**Geochemical Analyses**

Air-dried mud material was investigated by all methods further mentioned except for organic-petrographical analyses, for which the mud was freeze-dried. For determination of organic acids the freshly collected mud samples were immediately amended with 5% (v/v) of 10N NaOH to stop microbial activity. Water samples were collected by removal of the supernatant after centrifugation, followed by filtration. The samples for turnover measurements were immediately transferred into glass flasks, filled without headspace and once back in Baku lab, stored at 4°C. Organic geochemical parameters were determined on samples that were collected in pre-cleaned Teflon cups and stored in liquid nitrogen within a few hours after sampling.
Determination of anions

All water extracts were analyzed in replicate by ion chromatography with conductivity detection (ICS 3000, Dionex Corp.). For chromatographic separation of the anions the analytical column AS 11 HC (Dionex Corp.) was used at a temperature of 35 °C. The sample was eluted by KOH solution of varying concentration over time. The initial KOH concentration was 0.5 mM, maintained for 8 min. After 10 min, 15 mM KOH solution was reached and kept constant for 10 min. After 30 min analysis time, 60 mM KOH concentration was reached, followed by a rapid increase to 100 mM after 32 min. At 32 min analysis time, KOH concentration was again at the initial level of 0.5 mM and kept there for an additional 15 min to equilibrate the system. For quantification of organic acids (formate, acetate) and inorganic anions (F\textsuperscript{-}, Cl\textsuperscript{-}, Br\textsuperscript{-}, SO\textsubscript{4}\textsuperscript{2-}) standards containing all of the investigated compounds were measured in different concentrations every day. Standard deviation of sample and standard quantification is below 10 %.

Determination of Reduced Sulfur Species/Fractionated distillation

Solid reduced sulfur species were quantified separately based on the extraction scheme of (Zhabina and Volkov, 1978) with some modifications for the separation of elemental sulfur. The sample is placed into a cold distillation apparatus (Kallmeyer et al., 2004). In a first step 8 ml of 6 M hydrochloric acid is used to liberate the acid-volatile sulfur (AVS) fraction, comprising hydrogen sulfide and monosulfides. In the second step 16 ml of a 1 M chromous chloride (CrCl\textsubscript{2}) solution is added to the sample to liberate the chromium-reducible sulfur (CRS) fraction, comprising mainly pyrite and other disulfides.
In the third step 20 ml N,N-dimethylformamide is added to the sample to obtain the elemental sulfur fraction (ES).

A constant flow of nitrogen is used to strip the liberated hydrogen sulfide gas from the sample and quantitatively collect it in a trap filled with 7 ml of 5% (w/v) zinc acetate solution. For each fraction a fresh trap is used; the reagents are simply added to the existing slurry. Each distillation step takes two hours to ensure sufficient time for the reaction.

The zinc acetate from the traps interferes with the spectrophotometric sulfide quantification (Cline, 1969), therefore the precipitated zinc sulfide is separated by centrifugation, the clear supernatant carefully decanted off and the pellet resuspended in demineralised water for analysis.

**Potential Sulfate Reduction Rates (SRR)**

The samples used for quantification of potential SRR were collected during October 2008 from three different mud volcanoes: at Dd from a depth of approximately four meters, at B and BJ from the surface of an actively mud-emitting pool. For sulfate reduction rate quantification the mud samples were diluted with anaerobic saline solution (for composition see Table S1) in a 1:1 ratio (w/v) in a 500 ml Duran flask. The flask was pre-flushed with gas (N\(_2\)/CO\(_2\) 80/20) for 5 minutes before quickly scooping in the mud, followed by flushing for another 5 minutes before screwing on the cap. After determination of the exact volume of mud by weighing, anaerobic saline solution was added to the flask and the slurry stirred for one hour. Aliquots of 3 ml were dispensed anaerobically into 30 autoclaved 16 ml screw cap culture tubes using a syringe and a needle. A mixture of 6 volatile fatty acids (VFA; acetate, butyrate, lactate, propionate, pyruvate, succinate) and
ethanol, each with a final concentration of 1 mM were added to the remaining slurry. From this VFA-slurry again 3 ml each were dispensed into each of 15 anoxic autoclaved screw cap culture tubes. Because of the storage in the cold room the samples were preincubated at their respective incubation temperature for 24 or 48 hours and labeled “24” and “48” accordingly. The VFA-amended samples were preincubated for 48 hours and labeled “48-VFA”.

In order to avoid any sulfate limitation and thereby causing potential biases when comparing the microbial activity between the different sites, all samples were incubated with the same final sulfate concentration of 20 mM. A thermal gradient block (TGB) was used for experiments requiring different incubation temperatures (eg, Elsgaard et al., 1994; Sagemann et al., 1998). One end of the block was heated to 95°C while the other was cooled to 5°C resulting in a temperature gradient from 82.6 to 10.8°C. The linearity of the thermal gradient was checked after one day of equilibration with a digital thermometer. The positions of the culture tubes in the TGB were chosen to achieve a resolution of 5°C. All experiments were done in duplicates.

After 24 or 48 hours of preincubation, approximately 1 MBq (15 µl) of radioactive $^{35}$SO$_4^{2-}$ tracer was added to each sample with a syringe. A carrier-free tracer radiotracer stock solution was diluted with a saline solution containing the same major salts as the anoxic saline solution that was used to prepare the slurry. Incubation with the tracer lasted 8 hours and was terminated by adding 3 ml of 20% (w/v) zinc acetate (ZnAc) solution and immediate vortexing (Sagemann et al., 1998). The ZnAc-fixed slurry was poured into a 50 ml centrifuge tube. Remaining sediment was washed out of the tube with twice 3 ml 20% ZnAc. The sediment-ZnAc slurry was then centrifuged (5 min, 4500 g) and the supernatant
carefully decanted off. A small amount of supernatant was kept for quantification of the total radioactivity. The pellet was used for sulfate reduction rate measurements. The samples were processed according to the cold chromium distillation protocol of Kallmeyer et al. (2004). In short, the pellet was washed out of the tube with 20 ml dimethylformamide (DMF) into a three-neck round-bottom flask, then 8 ml HCl and 16 ml CrCl$_2$ solution was added. The liberated H$_2$S was flushed with N$_2$ into a 5% ZnAc trap. The radioactive sulfide was quantified using a Packard 2900 TR Tri-Carb scintillation counter. The sulfate reduction rate (SRR) was determined according to (Jørgensen, 1978). Control samples that were first fixed in ZnAC prior to radiotracer addition were processed together with the regular samples to quantify the background and to calculate the minimum detection limit.

**Microcosms to Test Sulfide and CO$_2$ production**

Mud and water samples were collected in 2007 from the D and B mud volcanoes. Experiments were carried out in glass tubes (20 ml) sealed with butyl-rubber stoppers and screw caps. Sediment samples (from 2007 trip) were mixed 1:1 with artificial mineral medium (after Widdel & Bak 1992; similar in composition to medium used for potential sulfate reduction rate measurements) to obtain homogenous slurries. Subsequently, 9 ml of medium were added to 3 ml of sediment slurry. All manipulations were performed under an atmosphere of nitrogen in an anoxic glove box. The headspace of the incubation tubes consisted either of methane (100%), air (100%) or of N$_2$/CO$_2$ (90/10 [v/v]; with CO$_2$ levels similarly unlimited as with gas composition used for potential sulfate reduction rate measurements). CO$_2$ was determined in all incubations using a GC 14B gas chromatograph.
(Shimadzu) as described in Nauhaus et al. (2002). Sulfide was determined in anaerobic incubations using the formation of copper sulfide (Cord-Ruwisch, 1985).

Gene quantification by qPCR

Mud and water samples were collected in 2007 from D (salse lakes D1 and D3 in 2008 data) and B (small salse within same B satellite visited in 2008) mud volcanoes. DNA extraction was carried out using a Fast DNA for Soil Kit (Fast DNA Spin Kit for soil, BIO 101, MP Biomedicals, Germany). To block sedimentary nucleic acid binding capacities, 10 µl of a 1% polyadenylic acid solution were added in the initial step (Webster, 2003). Directly before PCR, 125 µl of 0.3% bovine serum albumine (BSA) in ultra-pure water were added as blocking agent to the Taqman master mix (Applied Biosystems, Germany) or the SYBR green® master mix (Eurogenetec, Germany). A real-time PCR instrument (ABI Prism 7000, Applied Biosystems) was employed to determine the 16S rRNA gene copy numbers of Archaea (Takai, 2000) and Bacteria (Nadkarni, 2002).

Molecular biological determination of sulfur-cycling bacteria

DNA Extraction

Mud samples collected from D, B, BJ and P during October 2008 for molecular analyses were stored at approximately 20°C before and after room temperature shipment. DNA extractions were conducted using the MoBio Ultraclean soil kit following a previously published protocol (Orphan et al., 2001). Due to inconsistencies in mud viscosity, the following starting material was used from each sample: Ds, 500 µl; Dd 0.5 gm weight wet; B1, 250 µl; BJ, 0.5 gm weight wet; P, 50 µl.
PCR Amplification and Cloning

Unless otherwise noted, amplification reactions followed published PCR mixtures and conditions (Harrison et al., 2009) with 0.5µl of Hotmaster Taq polymerase (Eppendorf AG, Hamburg, Germany). Bacterial 16S rRNA genes were amplified from Dashgil (D2D) using bacterium specific forward primer BAC-27F and universal reverse primer U-1492R (Lane, 1991). Thermocycling conditions included an initial 94°C denaturating step for 2 minutes followed by 30 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute, and then a final 72°C elongation step for 6 minutes. The products of 2 reactions were pooled and cleaned using a Multiscreen HTS plate (Millipore). The resulting purified 16S rRNA gene amplicons were ligated into pGEM-T Easy vector and used to transform JM109 chemically competent cells (Promega, Stoughton, WI). Genus-specific 16S rRNA primers DEM116F and DEM1164R were used to check for presence of Desulfotomaculum (Stubner and Meuser, 2000). Thermocycling conditions included an initial 94°C denaturation step for 1 minute followed by 40 cycles of 94°C for 45 seconds, 57.5°C for 45 seconds and 72°C for 1 minute, and then a final 72°C elongation step for 6 minutes.

An equimolar mix of forward primers DSR1F, DSR1Fa, DSR1Fb, DSR1Fc, and DSR1Fd and reverse primers DSR4R, DSR4Ra, DSR4Rb, DSR4Rc, DSR4Rd, and DSR4Re (Zverlov et al., 2005) was used to amplify the 1.9 kb dsrAB fragment from Dashgil (D2D). The 1.9 kb PCR product was excised from a 1% agarose gel and purified using a Quiaquick Gel Extraction kit (Qiagen Corp., Valencia, CA). Resulting DsrAB amplicons were ligated into pGEM-T Easy vector and used to transform JM109 chemically competent cells (Promega, Stoughton, WI).
The primer set AprA-1-FW/AprA-5-RV was used to amplify the approximately 0.4 kb fragment of the aprA gene (Meyer and Kuever, 2007a) from 2 µl of Dd and BJ and 1 µl of Ds, B and P DNA extractions. Thermocycling conditions included an initial 94°C denaturation step for 3 minutes followed by 40 cycles of 94°C for 30 seconds, 54°C for 55 seconds and 72°C for 1 minute, and then a final 72°C elongation step for 6 minutes. The products (1 reaction each from Dd, Ds, B and P templates, and 2 pooled reactions from the BJ template) were cleaned using a Multiscreen HTS plate (Millipore). The resulting purified aprA gene amplicons were ligated into pGEM-T Easy (D2D and S1; Promega) or pCR 4.0 TOPO TA (Ds, B and P; Invitrogen Corp., Carlsbad, CA) vectors and used to transform JM109 (Dd and BJ; Promega, Stoughton, WI) or One-Shot TOP10 (Ds, B and P; Invitrogen Corp., Carlsbad, CA) chemically competent cells according to the manufacturer’s instructions.

Sequencing and Phylogenetic Analysis

HaeIII restriction fragment length polymorphism (RFLP) analysis was performed on products amplified with the standard primer set M13F/M13R (Pernthaler et al., 2008; Harrison et al., 2009; New England Biolabs, Ipswich, MA) from all 16S rDNA, dsrA and aprA clones. One RFLP pattern from Ds had representative clones from two distinct phylogenetic groups; all clones from with this pattern were further digested with PstI (New England Biolabs, Ipswich, MA) and the pattern divided accordingly. From the Dashgil_D2D samples, 62 dsrA clones were screened (11 unique RFLP patterns), and 74 bacterial 16S rDNA clones were screened (11 unique RFLP patterns). The following number of aprA clones were retrieved (with number of unique RFLP patterns in parenthesis) from each
TMV: Ds: 89 (16), Dd: 47 (9), B: 69 (14), BJ: 76 (6), P: 93 (21). Representative clones with unique restriction patterns were cleaned using Multiscreen HTS plates (Millipore) and sequenced unidirectionally either in house with a CEQ 8800 capillary sequencer according to the DTCS protocol (Beckman Coulter, Fullerton, CA), or at the ASGPB DNA Sequencing Facility of the University of Hawai’i at Manoa. All sequences were manually edited using Sequencher 4.5 software (Gene Codes, Ann Arbor, MI) and closest relatives in the GenBank database were identified using BLASTN (Altschul et al., 1997). All aprA sequences (131 translated amino acid characters each) were manually aligned using the ARB software package (version 7.12.07org, ARB_EDIT4; Ludwig et al., 2004) into an alignment of full-length aprA SRP and SOB reference strains and closest relatives recovered from the public databases. The aligned sequences were then added to the existing full length aprA tree using the quick add maximum parsimony method with a filter to mask regions outside of the 131 amino acid characters. Genbank accession numbers for aprA, dsrA and 16S rRNA sequences are JX908299-JX908360 and JX889577-JX889588.

ACKNOWLEDGEMENTS

We would like to acknowledge Dr. Chingiz Aliyev and Rauf Bagirli for assistance during field trip and laboratory investigations in Baku; Daniela Zoch and Holger Probst for technical work at BGR, and Andrea Vieth-Hillebrandt and Kristin Günther for water analysis at Helmholtz Centre Potsdam GFZ. Funding for this work was provided by a DOE Career grant (to VJO), a NSF GRFP (to AG-S) and through the Forschungsverbund GeoEnergie of the German Ministry for Education and Research (BMBF, to JK and PS).
REFERENCES


Dimitrov, L.I. (2002). Mud volcanoes--the most important pathway for degassing deeply buried sediments. *Earth-Science Reviews* 59, 49-76.


sulfuroxidizing prokaryotes. Microbiology-SGM 153, 3478-3498.


**Figures and Tables**
Figure 1. Location of terrestrial mud volcanoes in Azerbaijan sampled in 2007 and 2008.
Figure 2. Potential sulfate reduction rates for A) Dashgil (Dd), B) Bakhar (gryphon sample B) and C) Boransyz-Julga (gryphon sample BJ). Rate measurements were taken on a thermal gradient block ranging from approx. 10°C to 82°C, with 3 incubation treatments: 24-hour preincubation, 48 hour preincubation, and 48 hour preincubation with volatile fatty
acids (VFAs). The dashed line indicates the minimum detection limit was 0.12 nmol/cm³/d, which is the average of all blank measurements plus 3 times the standard deviation.
Figure 3. Phylogenetic relationships of A) putative sulfur-oxidizing bacterial and B) putative sulfate-reducing bacterial aprA sequences retrieved from Dashgil (Dd, Ds; 89 and 47 clones, respectively), Bakhar (B; 69 clones), Boransyz-Julga (BJ; 76 clones) and Perekyushkul (P; 93 clones) mud volcanoes sampled in 2008 and inferred via maximum parsimony using the ARB software package. The number of clones represented by each
OTU sequence in the tree is listed in parenthesis after the name. The scale bar corresponds to 10% estimated sequence divergence.
SUPPLEMENTAL ONLINE MATERIAL

RESULTS

Geochemical characterization

The organic matter (OM) of all samples appears to be a mixture of marine and terrestrial material, sourced from sediments with a thermal maturity of around 0.5 % vitrinite reflectance. The total organic carbon (TOC) composition within the 4 mud volcanoes ranged between 0.58 and 1.5% with $d^{13}C$ values between -25.2‰ down to -26.7‰. With the exception of the salse lake at D (D3), $d^{13}C_{org}$ for the coastal mud volcanoes (D and B) were approximately 1‰ heavier (avg. -25.2‰) compared with the inland sites (P and BJ (avg. -26.5‰; Tables 1, S4). Thermal maturity for the majority of samples had a $T_{max}$ around 420 to 430°C, with the exception of B, which showed slightly lower $T_{max}$ values (392°C; Tables 1, S4). B samples also exhibited strong degradation of metabolizable OM, with a loss of n-alkanes and occurrence of an unresolved complex
mixture (UCM). These samples were also characterized by lower contents of TOC (< 0.7 % TOC) in comparison with the other volcano samples (1.2 to 1.3 % TOC on average), and an obvious type II signal in the Rock Eval programs. Based on the thermal maturity, the organic material of the B volcano may have originated from a shallower source relative to the other three volcanoes in this study. Rock Eval programs exhibited relatively higher S1 signals in B and BJ samples indicating free bitumen in the rock matrix (Tables S4, S5). Organic acids in the pore fluids (formate and acetate) were detected in all but D (D1) and ranged between 14 to 49 µM. The carbonate fraction of the muds in the BJ and P volcanoes were characterized by heavy δ^{13}C (1.4 to 3.0‰) and δ^{18}O (-2.4 to -2.9‰) values relative to carbonates from D and B (δ^{13}C: 0.28 to -2.4‰ and δ^{18}O: -2.5 to -4.3‰).

Indirect evidence of methanogenesis was found in stable isotope analyses from inland mud volcanoes, P and B, with enriched δ^{13}C values of carbonate suggestive of carbonate precipitation under methanogenic conditions (Feyzullayev and Movsumova, 2010).

**In situ detection of archaeal sulfate-reducing consortia by epifluorescent microscopy**

Due to high viscosity/hydrocarbon content of the mud samples, CARD-FISH hybridization reactions were only successful with some of the samples. In the D1 sample, cell aggregations consisting of ANME-2 archaea and SRB associated with the Desulfosarcina/Desulfococcus group (Figure S2) and members of the Desulfobulbaceae (data not shown) were detected. These aggregates were also detected in shallow Ds and BJ2 samples, and single cells staining only with DAPI were visible in all samples (data not shown). Aggregates ranged in diameter from 3 µm to 8 µm with three distinct
morphologies similar to those previously observed in cold seeps: ANME core with SRB shell, adjoined clumps or a heterogeneous mixture of both (e.g., Orphan et al., 2002).

**EXPERIMENTAL PROCEDURES**

**Geochemical Analyses**

**Analysis of the stable isotope composition**

Total organic carbon (TOC) and δ\(^{13}\)C\(_{\text{org}}\) as well as total nitrogen (TN), δ\(^{15}\)N and total carbon (TC) were determined using an elemental analyzer (NC2500 Carlo Erba) coupled with a ConFlowIII interface on a DELTAaplusXL mass spectrometer (ThermoFischer Scientific) at the Deutsches GeoForschungsZentrum in Potsdam, Germany. The isotopic composition is given in delta notation relative to a standard:

\[
d(\text{%}) = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}}\right) \times 1000
\]

The isotopic ratio (R) and standard for carbon is \(^{13}\)C/\(^{12}\)C and VPDB (Vienna PeeDee Belemnite), respectively, and for nitrogen is \(^{15}\)N/\(^{14}\)N and air.

The TOC contents and δ\(^{13}\)C\(_{\text{org}}\) values were determined on decalcified samples. Around 3 mg of sample material were weighed into Ag-capsules, dropped with 20% HCl, heated for 3 h at 75°C, and finally wrapped into the Ag-capsules and measured as described above. The calibration was performed using elemental (Urea) and certified isotope standards (USGS24, CH-7) and proofed with an internal soil reference sample (Boden3). The reproducibility for replicate analyses is 0.2% for TOC and 0.2‰ for δ\(^{13}\)C\(_{\text{org}}\).

For total carbon, nitrogen and δ\(^{15}\)N determination, around 20 mg of sample material was loaded in tin capsules and combusted in the elemental analyzer. Total carbon and
nitrogen content were calibrated against Acetanilide whereas for the nitrogen isotopic composition two ammonium sulfate standards (e.g., IAEA N-1 and N-2) were used. Replicate determinations show a standard deviation less than 0.2% for C and N and 0.2‰ for $\delta^{15}$N.

The stable isotope compositions of carbonates ($\delta^{13}$C and $\delta^{18}$O) were determined in continuous flow mode using a Finningan GasBenchII with carbonate option coupled to a DELTA$^{+}$XL mass spectrometer. From each sample, about ~ 250 mg were loaded into 10 ml Labco Exetainer vials. After automatically flushing with He, the carbonate samples were reacted in phosphoric acid (100 %) at 75°C for 60 min, following the analytical procedure described in Spötl and Vennemann (2003). The $\delta^{18}$O values refer to Vienna-Standard Mean Ocean Water (SMOW), and the $\delta^{13}$C values to the Cretaceous Pee Dee Belemnite (PDB). The isotope compositions were given relative to the VPDB standard in the conventional delta notation, and were calibrated against two international reference standards (NBS 19 and NBS18). The standard deviation for reference analyses was 0.06% for $\delta^{13}$C and 0.08‰ for $\delta^{18}$O.

**Rock Eval pyrolysis**

Powdered samples were analyzed for organic carbon content (TOC, after acidification of samples to remove carbonate) using a Leco CS-244 analyzer. Pyrolysis measurements were performed using a Rock-Eval 6 instrument, using the following temperature profile: Start at 300 °C (3 min isothermally), then heated for 25 °C/min up to 650 °C (0 min.). Jet-Rock 1 was run as standard and checked against the acceptable range given in NIGOGA (Norwegian Industry Guide to Organic Geochemical Analyses).
Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH)

Mud samples collected during October 2008 for in situ analyses were fixed in 2% formaldehyde for approximately 1.5 hours at room temperature, washed twice with phosphate-buffered saline (PBS; Pernthaler et al., 2008), once with 1:1 PBS: ethanol, resuspended in 100% ethanol and stored at approximately -20°C before and after room temperature shipment. For CARD-FISH analyses, 50 µl mud collected from D1 was brought to 1.5 ml in a TE (10 mM Tris-HCl and 1 mM EDTA (pH 9.0)), 0.01 M pyrophosphate solution, heated in a histological microwave oven (Microwave Research and Applications) for 3 minutes at 60°C, cooled to room temperature and incubated in 0.1% hydrogen peroxide for 10 minutes. The solution was then sonicated on ice for two 5 second bursts with a Vibra Cell sonicating wand (Sonics and Materials, Danbury, CT) at an amplitude setting of 30 and overlaid on a Percoll density gradient (Orphan et al., 2002). Resulting filters were permeabilized in sequential HCl, SDS and lysozyme solutions as described by Pernthaler et al. (2004). Horseradish peroxidase-labeled probes (Biomers) targeting Desulfobacteraceae (DSS_658, 50% formamide hybridization buffer; Manz et al., 1998) and anaerobic methane-oxidizing archaeal clade ANME-2 (Eel_MS_932, 45% formamide hybridization buffer; Boetius et al., 2000) were then used in a dual-hybridization CARD-FISH reaction (Pernthaler et al., 2008). The first hybridization reaction took place in a histological microwave oven (Microwave Research and Applications) for 30 minutes at 46°C, followed by an amplification reaction using fluorescein-labeled tyramides. The second hybridization reaction was carried out in a
hybridization oven for 2.5 hours at 46°C followed by an amplification reaction using Alexa Fluor 546-labeled tyramides. Micrograph images were taken with a Deltavision RT microscope system (Applied Precision; Pernthaler et al., 2008).

SUPPLEMENTAL REFERENCES


Figures and Tables

Figure S1. Photographs taken in October 2008 of A) Boransyz-Julga gryphon and B) Dashgil salse lake.
Figure S2. Putative methane-oxidizing aggregates from Dashgil (D1) mud samples identified by CARD-FISH after hybridization with oligonucleotide probes specific to anaerobic methane-oxidizing archaea ANME-2 (EelMsMX_932; red), and Desulfosarcina-Desulfococcus members (DSS_658; green). The general DNA stain DAPI is shown in blue. Scale bar = 2 µm.

Table S1. Composition of the synthetic saline solution; the solutions were added after the anoxic cooling post autoclaving

<table>
<thead>
<tr>
<th>Add after anoxic cooling post autoclaving:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml trace elements mixture</td>
<td>50 ml H₂O + 2.1 g FeSO₄·7H₂O + 13 ml HCl (25%), 30 mg H₂BO₃, 100 mg MnCl₂·4H₂O, 190 mg CoCl₂·6H₂O, 24 mg NiCl₂·6H₂O, 2 mg CuCl₂·2H₂O, 144 mg ZnSO₄·7H₂O, 36 mg Na₂MoO₄·2H₂O per Liter, autoclaved</td>
</tr>
<tr>
<td>2 ml SeW-solution</td>
<td>200 mg NaOH, 6 mg Na₂SeO₃·5H₂O, 8 mg Na₂WO₄·2H₂O per Liter, autoclaved</td>
</tr>
<tr>
<td>60 ml 1 M NaHCO₃-solution</td>
<td>84 g NaHCO₃ per Liter, anoxic, autoclaved</td>
</tr>
<tr>
<td>2 ml 7-vitamin mixture</td>
<td>100 ml H₂O + 4 mg 4-amino benzoic acid, 1 mg D(+)‐biotin, 10 mg nicotinic acid, 5 mg Ca-D(+)‐pantothenat, 15 mg pyridoxine hydrochloride, 10 mg thiamine chloride hydrochloride, filtered sterile</td>
</tr>
<tr>
<td>2 ml B₁₂-solution</td>
<td>5 mg cyan cobalamin in 100 ml H₂O, filtered sterile</td>
</tr>
</tbody>
</table>
Table S2. Closest relatives of phylotypes retrieved from A.) 16S rRNA and B.) *dsrA* gene clone libraries of Dashgil sample (deep pool Add). *env.* = *environmental sequence*

### A.

<table>
<thead>
<tr>
<th>Classification</th>
<th>clone (frequency)</th>
<th>Accession</th>
<th>Description</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>D12 (2)</td>
<td>JF806919</td>
<td>env., river</td>
<td>98%</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>A10 (5)</td>
<td>HQ183982</td>
<td>env., landfill leachate pond sediment</td>
<td>98%</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>B6 (4)</td>
<td>HQ397021</td>
<td>env., landfill leachate pond sediment</td>
<td>97%</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>C8 (20)</td>
<td>HQ689286</td>
<td>env., landfill leachate pond sediment</td>
<td>96%</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>G4 (1)</td>
<td>HQ183982</td>
<td>env., landfill leachate pond sediment</td>
<td>99%</td>
</tr>
<tr>
<td>Delta-proteobacteria</td>
<td>B4 (3)</td>
<td>FJ517079</td>
<td>env., wetlands</td>
<td>94%</td>
</tr>
<tr>
<td>Delta-proteobacteria</td>
<td>F1 (2)</td>
<td>JF806819</td>
<td>env., river</td>
<td>99%</td>
</tr>
<tr>
<td>Delta-proteobacteria</td>
<td>H2 (1)</td>
<td>NR_044429</td>
<td><em>Geobacter odorans</em> strain RedI</td>
<td>99%</td>
</tr>
<tr>
<td>Delta-proteobacteria</td>
<td>G2 (1)</td>
<td>AF177428</td>
<td>env., oil reservoir</td>
<td>95%</td>
</tr>
<tr>
<td>Epsilon-proteobacteria</td>
<td>A1 (39)</td>
<td>JF806914</td>
<td>Uncultured <em>Sulfurovum</em> sp.</td>
<td>86%</td>
</tr>
<tr>
<td>Epsilon-proteobacteria</td>
<td>G1 (5)</td>
<td>JF806947</td>
<td>Uncultured <em>Sulfurovum</em> sp.</td>
<td>89%</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>C12 (4)</td>
<td>GQ848205</td>
<td>env., soda lake</td>
<td>93%</td>
</tr>
</tbody>
</table>

### B.

<table>
<thead>
<tr>
<th>Classification</th>
<th>clone (frequency)</th>
<th>Accession</th>
<th>Description</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desulfobacteraceae</td>
<td>B2 (29)</td>
<td>EU496587</td>
<td>env., methane-ooze sediment</td>
<td>82%</td>
</tr>
<tr>
<td>Desulfobacteraceae</td>
<td>A3 (15)</td>
<td>AF482455</td>
<td><em>Desulfobacterium annulatum</em></td>
<td>79%</td>
</tr>
<tr>
<td>Desulfobacteraceae</td>
<td>D1 (4)</td>
<td>AY626030</td>
<td><em>Desulfurobacterium</em> <em>thornii</em> strain DSM 9680</td>
<td>76%</td>
</tr>
<tr>
<td>Desulfobacteraceae</td>
<td>E1 (1)</td>
<td>AY626031</td>
<td><em>Desulfurobacterium</em> <em>lincolns</em> strain DSM 2076</td>
<td>75%</td>
</tr>
<tr>
<td>Desulfobacteraceae</td>
<td>G4 (1)</td>
<td>CP001940</td>
<td><em>Desulfurobacterium</em> <em>alkaliphilus</em> AHT2</td>
<td>89%</td>
</tr>
<tr>
<td>F7 (3)</td>
<td>AB124917</td>
<td></td>
<td>Uncultured sulfate-reducing bacteria, hydrothermal vent</td>
<td>87%</td>
</tr>
<tr>
<td>H2 (5)</td>
<td>FJ48851</td>
<td></td>
<td>Uncultured sulfate-reducing bacteria, estuarine sediment</td>
<td>82%</td>
</tr>
<tr>
<td>H7 (3)</td>
<td>JF950248</td>
<td></td>
<td>Uncultured bacteria, anoxic fjord sediments</td>
<td>77%</td>
</tr>
<tr>
<td>H4 (1)</td>
<td>FJ748848</td>
<td></td>
<td>Uncultured sulfate-reducing bacteria, estuarine sediment</td>
<td>77%</td>
</tr>
</tbody>
</table>
Table S3. Archaeal and Bacterial 16S rRNA gene abundance from D (Dashgil) and B (Bakhar) samples collected in 2007.

<table>
<thead>
<tr>
<th>Sample, depth collected</th>
<th>Total Archaea</th>
<th>Total Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dashgil (D1) big saline lake, 5.8m</td>
<td>2.99E+07 +/-</td>
<td>1.81E+07 +/-</td>
</tr>
<tr>
<td>Dashgil (D1) big saline lake, 0.5m</td>
<td>7.62E+07 +/-</td>
<td>5.12E+07 +/-</td>
</tr>
<tr>
<td>Dashgil (D3) small saline lake, 6.5m</td>
<td>8.00E+08 +/-</td>
<td>8.74E+07 +/-</td>
</tr>
<tr>
<td>Dashgil (D3) small saline lake, 0.2m</td>
<td>1.97E+08 +/-</td>
<td>7.65E+07 +/-</td>
</tr>
<tr>
<td>Bakhar small saline lake, 4.2m</td>
<td>1.30E+07 +/-</td>
<td>6.06E+06 +/-</td>
</tr>
<tr>
<td>Bakhar small saline lake, 0.2m</td>
<td>2.11E+07 +/-</td>
<td>2.66E+06 +/-</td>
</tr>
</tbody>
</table>

Table S4. Geochemical characteristics of terrestrial mud volcanoes of Azerbaijan sampled in October 2008. *n/a = not available*

<table>
<thead>
<tr>
<th>Mod Volcano</th>
<th>Geographic Setting</th>
<th>Coordinates</th>
<th>Sample Name and Feature</th>
<th>Temperature, °C (measurement depth, m)</th>
<th>D1: Saline Lake</th>
<th>D3: Saline Lake</th>
<th>D4: Gryphon</th>
<th>P1: Perekhylshul</th>
<th>B1: Beremysz-Julga</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1: Dashgil</td>
<td>Proximal to Caspian Sea Quaternary Sediments</td>
<td>N 36° 50' 47.8&quot; E 40° 29' 05.2&quot;</td>
<td>D1: Saline Lake</td>
<td>19.3 (0.2), 18.7 (5.5)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D3: Saline Lake</td>
<td>19/20</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D4: Gryphon</td>
<td>16.3 (6.2)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**Geochemistry**

- **Fluoride** (mM) 0.18 0.14 n/a n/a
- **Sulfate** (mM) 0.75 <0.16 0.47 0.32 <0.05
- **Chloride** (mM) 378 264 209 86 64
- **Nitrate (mM)** 0.82 0.97 0.84 0.16 0.16
- **Acetate** (mM) <0.010 <0.007 <0.020 <0.009 <0.016
- **Formate** (mM) <0.013 <0.027 <0.002 <0.002 <0.024

**Stable Isotope Composition**

- **d15N** 2.2 2.5 2.0 2.7 3.0
- **d2H** 0.09 0.09 0.10 0.11 0.09
- **d18O** 1.55 2.74 2.67 2.41 1.77
- **d34S** 1.06 0.85 0.95 1.29 0.71
- **d13C** -25.1 -26.4 -28.9 -26.6 -26.3
- **CaCO3 (%)** 12.5 16.3 14.4 9.3 8.8
- **d13Corg (%)** 0.42 -0.59 -1.04 -2.71 4.82
- **d34Sorg (%)** -3.47 -2.50 -4.03 -2.41 -1.23

**Organic Analysis**

- **S1, S2, S3 (mg%)** 0.42, 2.22, 1.01 0.12, 1.46, 1.21 0.33, 2.62, 0.71 0.08, 0.87, 1.26 0.24, 2.16, 1.51
- **Tmax (°C)** 431 431 433 431 428
- **Hydrogen Index III (mg) 193 150 224 216 214
- **Oxygen Index O1 (mg C-O1g TOC)** 88 124 61 82 159
- **TOC (%)** 1.15 0.97 1.17 1.54 1.01

**Reduced Sulfur Species**

- **(AVS,Grx,DM)** n/a 0.01 0.01 0.08 0.00 0.95 0.05 n/a n/a

**Activity**

- **(AVS,Grx,DM)** 27.68 72.38 0.02
Table S5. Geochemical characteristics of four terrestrial mud volcanoes of Azerbaijan sampled in October 2008. g/a = not available

<table>
<thead>
<tr>
<th>Mud Volcano</th>
<th>Geographic Setting</th>
<th>Coordinates</th>
<th>Sample Name and Feature</th>
<th>Temperature, °C (measurement depth, m)</th>
<th>POREWATER GEOCHEMISTRY</th>
<th>STABLE ISOTOPE COMPOSITION</th>
<th>ORGANIC ANALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>D: Dashgil</td>
<td>Proximal to Caspian Sea Quaternary Sediments</td>
<td>N 39° 59' 47.8&quot; E 49° 24' 08.8&quot;</td>
<td>Ds: Pool (surface) 17.2 (0.2), Dh: Pool (deep) 20.6 (1.0), 20.8 (1.25)</td>
<td>Fluoride (mM) 0.03, Chloride (mM) 312, Bromide (mM) 0.63</td>
<td>Na, wt. % 2.0, Ca, wt. % 2.93, TOC, wt. % 1.50, d13Corg, % -25.6, CaCO3, calc % 11.9, d18OCaCO3, % 0.20, d18Owater, % -4.02</td>
<td>Tmax (°C) 423, TOC (%) 1.63</td>
<td></td>
</tr>
</tbody>
</table>