Long term sustainability of a high energy, low diversity crustal biome

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One sentence summary: **Nearly 3 km deep in the Earth's crust, a simple bacterial community has survived for millions of years on geological sources of hydrogen and sulfate**

Abstract

Geochemical, microbiological and molecular analyses of alkaline, saline groundwater at 2.8 km depth in Archaean metabasalt revealed a microbial biome dominated by a single phylotype affiliated with thermophilic sulfate reducers belonging to *Firmicutes*. These sulfate reducers were sustained by geologically-produced sulfate and hydrogen at concentrations sufficient to maintain activities for millions of years with no apparent reliance upon photosynthetically-derived substrates.

Most subsurface microbial ecosystems examined to date (including subseafloor sediments, deep sea hydrothermal vents, terrestrial sedimentary aquifers and petroleum reservoirs), ultimately depend on sunlight. These studies have been mostly confined to less than 1 km depth and are either supported by photosynthetically-produced electron donors and acceptors transported by groundwater or seawater with ages much less than a million years, or in constant contact with oxygenated seawater migrating through the underlying fractured basaltic aquifer (*1-4*). Although two occurrences of autotrophic microbial communities have been reported to exist in <300 m deep volcanic aquifers flushed with fresh, meteoric water (*5, 6*), their long term sustainability on H_2 and isolation from photosynthetically-produced substrates have not been demonstrated. While the existence of subsurface microorganisms at depths greater than 1 km in pristine environments is well established (*7*), much still remains unknown regarding the abundance, diversity and sustainability of these microbial communities over geological time scales.

To determine the long-term sustainability of a deep terrestrial environment we examined the microbial diversity and metabolic activity of a 3-4 km-deep fracture in 2.7 Ga Ventersdorp Supergroup metabasalt for which fracture water ages of tens of millions of years (*8*), abundant abiogenic hydrocarbons (*9*), and radiolytically-produced H_2 (*10*) have been reported. To characterize the indigenous microbial composition and its principal respiratory pathway, we analyzed 16S rRNA genes, aqueous and gas geochemistry, and stable and noble gas isotopic signatures of moderately saline groundwater emanating from a fracture zone at 2.825 kilometers below land surface (kmbls) in the Mponeng Au mine, South Africa. This high-pressure, water-bearing fracture was intersected during exploratory drilling ahead of a tunnel advancing into an unmined zone ~100 m above the

Ventersdorp Contact Reef (VCR) ore zone. The fracture water was initially sampled as soon as it was safe to do so (4 days after the fracture intersection), and 3 subsequent samples were obtained over a 54-day interval (Table 1) to monitor drilling contamination and possible changes of community structure and geochemistry as the fracture was dewatered and prior to sealing by the mine's cementation team.

Fracture water samples yielded a uniform community structure dominated by a single phylotype (MP104-0916-b1) that comprised >88% of the clones in 16S rDNA libraries, but did not appear in the water used for mining (Fig. 1a). This phylotype was related (96% similarity) to an uncultured clone recovered from thermal fluids of oceanic crust (*11*) or *Desulfotomaculum kuznetsovii* (91% similarity) (*12*), a sulfate reducer growing at moderately thermophilic conditions. Of the other minor bacterial and archaeal phylotypes associated with the fracture water, MP104-1109-a19 resembled (98% similarity) environmental clones (SAGMEG-2 group) obtained from fracture water in an adjacent mine (*13*) (Fig. 1b). Other phylotypes were closely related (98 - 99% similarity) to environmental clones recovered from surface environments (such as soils and sludge) or to various mesophiles distributed within *Proteobacteria*, *Firmicutes*, and *Methanobacteria* (Fig. 1). These minor phylotypes did not resemble those from the mining water (supporting online material; SOM).

High-density 16S rDNA microarray analysis was also used as a more sensitive approach to assess microbial diversity. Array hybridization results supported the finding that microbial diversity was much less (Table S2) and the *Firmicutes* were of greater relative abundance (Table S3) in the fracture water than the mining water. Some overlap

between the sequences found in the fracture water and those of the mining water is expected because mining water is a mixture of recycled fracture water and surface water.

The geochemistry of the fracture water was characterized to identify its origin and to assess the principal metabolic pathways (Tables 1, S1 and S4). Over the observation period, the temperature decreased from >60°C to 52°C; while the pH was constant and the redox potential (Eh) increased from –330 to –263 mV. The Cl^-, Br^- and SO_4^{2-} concentrations increased significantly, whereas other anion concentrations fluctuated or decreased slightly. Reduced gases (H_2 and CH_4) were abundant (> 1 mM) and their concentrations increased as water flow rates declined and gas flow rates remained constant. Formate and acetate ranged from 7 to 9 μM and 22 to 36 μM, respectively. The δ^2H and $\delta^{13}C$ of the abundant, dissolved C_{1-4} hydrocarbons indicated an abiogenic origin based on their similarity to abiogenic hydrocarbon gases identified at other Precambrian Shield sites (*9*). The δ^2H and $\delta^{18}O$ values of the fracture water, –29.1‰ and –6.9‰ VSMOW (Vienna Standard Mean Ocean Water), respectively, plotted slightly above the global and local meteoric line (*14*). The δ^2H-H_2 ranged between –680 and –690‰ VSMOW, which when compared with the δ^2H-H_2O yielded isotopic equilibrium temperatures of 62°C for the first sample declining to 49°C for the last sample. Based upon the thermal conductivity model (*15*) and the heat flow data (*16*), the initial temperature equates to that at 4.2 kmbls and the final temperature to that at 2.9 kmbls.

Noble gas analyses yielded elevated radiogenic and fissiogenic concentrations of ^4He, ^{40}Ar, ^{134}Xe and ^{136}Xe (Table S1) but exhibited no systematic trend during the observation period. The bulk model age of the fracture water ranged from (15.8±7.9) Ma to (25.0±3.8) Ma (Table 1). This model age represents either the true subsurface residence

time or the mixing between an ancient (0.8 to 2.5 Ga), saline, H_2, C_{1-4} and sulfate-rich, hydrothermal fluid with a ~3 to 4 Ma old, moderately saline, H_2, C_{1-4} and sulfate-poor, paleometeoric water (SOM).

The exceptional dominance of the *Firmicutes* clones, distinct δ^2H and $\delta^{18}O$ values of the fracture water from those of the mining water (*17*), and the increasing H_2, CH_4, He, Cl^-, Br^- and SO_4^{2-} concentrations during the observation period suggests that the fracture environment favors their survival and that mining operations had minimal impact on this environment during depressurization and dewatering. *Desulfotomaculum*-related environmental clones and isolates have been detected from other subsurface environments, including an oil reservoir (*18*), deep sedimentary strata (*19, 20*), an ore deposit (*21*), and saline water emanating from a 3.2-kmbls borehole located 13 km east of this borehole (*22*). The evident success of these microorganisms may not be surprising since their ability to form endospores would facilitate their survival during periods of low water activity, nutrient deprivation, and sub-optimal temperature (*23*). Whether the less abundant members of the clone libraries represent indigenous microorganisms that are capable of acquiring energy through metabolisms distinct from those strains they most resemble phylogenetically, or alternatively represent moribund relics of shallower, less saline paleometeoric water that has mixed with this fracture water is not known.

Sulfur isotopic analyses yielded $\delta^{34}S$ values of HS^- ranging from 11‰ to 13‰ Vienna Canon Diablo Meteorite (VCDT), and those of SO_4^{2-} from 19‰ to 26‰ VCDT (Table S4). The depleted $\delta^{34}S$ value of HS^- relative to that of SO_4^{2-} (-7.7‰ for sample #4 and -12.4‰ for sample #7) is consistent with microbial sulfate reduction. The fact that SO_4^{2-} concentrations in the fracture water were higher than that of the overlying dolomitic

aquifer (*13*), and that SO_4^{2-} has been reported in analyses of fluid inclusions from hydrothermal quartz in the VCR (*24*) and the reservoir mixing-fractionation model (SOM) all suggest that SO_4^{2-} originated from the ancient hydrothermal fluid, not the paleometeoric end member. The $\delta^{34}S$ values of fracture pyrite (0‰ to 2‰ VCDT) were less than that of the coexisting barite (10.1‰ VCDT) (Table S4) and were consistent with those of hydrothermal 2.0 Ga pyrite from the VCR in the same mine (*25*). The $\Delta^{33}S$ values for all samples clustered around 0‰ (Table S4). To reproduce the $\Delta^{33}S$ of total dissolved S species (-0.022‰) for sample #7, a mixing of sulfate derived from pyrite oxidation and barite dissolution with a Rayleigh isotopic fractionation by microbial sulfate reduction was required (SOM). Such a mixture comprised of 30% SO_4^{2-} derived from oxidation of pyrite by radiolytically-produced oxidants (*10*) and 70% SO_4^{2-} from the dissolution of barite and produced an initial $\delta^{34}S$ value of 8.13‰ VCDT and a $\Delta^{33}S$ value of -0.0087‰ (SOM). The isotopic evolution from initial 8.13‰ VCDT to the present observation (18.63‰ VCDT for total dissolved S species) requires a Rayleigh fractionation process where 70% of the initial SO_4^{2-} was removed as microbially-precipitated pyrite (SOM). By this model the total HS^- formed by microbial sulfate reduction would be 4.35 mM (1.52 mM × 70% / 30% + 0.80 mM HS^-; SOM).

The Gibbs free energy for microbial redox reactions was calculated to provide additional constraints on the dominant respiratory pathway occurring in this fracture water. Sulfate reduction dominated the free energy yields for a wide range of electron donor and acceptor combinations (Table 2). The free energy yields for these reactions were much greater than the minimum requirement for synthesis of 1/3 ATP molecule by pumping one proton across the membrane (~-20 kJ) (*26*). Among electron donors available for sulfate

reduction and methanogenesis, H_2 yielded more free energy than acetate and formate, and H_2-utilizing sulfate reduction yielded the highest free energy and energy flux (Table 2), suggesting that CO_2-utilizing methanogens and acetogens cannot sustain as high a population density as sulfate reducers and therefore would be minor constituents of the community, as is observed in the clone libraries. The prominence of the free energy flux for H_2-utilizing sulfate reduction over other metabolic reactions is consistent with the depletion of $\delta^{34}S$ values of HS^- relative to SO_4^{2-} (*27*) and the physiological characteristics inferred from the dominant phylotype.

The in situ rate of microbial sulfate reduction was estimated to range from 0.22 to 1.45 nM yr^{-1}, or from 5.5×10^{-18} to 3.6×10^{-17} moles $cell^{-1}$ yr^{-1} (assuming all observed cells, 4×10^7 cells L^{-1}, actively reduce SO_4^{2-}) based upon the potential microbially-produced sulfide (4.35 mM; SOM) and fracture water residence time of ~3 or ~20 Ma inferred from noble gas analysis. Such a rate is comparable to rates reported in subseafloor sediments (*28*) and to the estimated rate of radiolytic H_2 generation (*10*), but far less (4 to 5 orders of magnitude) than the maintenance energy demand of mesophilic sulfate reducers as determined in laboratory experiments (*29*) (SOM). The estimated in situ sulfate reduction rate when combined with the experimental maintenance energy demand (48 moles (g dry wt. cell)$^{-1}$ yr^{-1}) (*29*) and assuming a 20 femtograms $cell^{-1}$ would only support 200 to 1600 cells L^{-1}, which is far below the observed ~4×10^7 cells L^{-1}. If cells were constantly growing and dying, then the experimental sulfate reducer yield of 12.2 g dry wt. $mole^{-1}$ (*29*) when combined with the in situ sulfate reduction rate would correspond to cell turnover times of 45-300 years. The isotopic estimate of the long-term in situ microbial activity, however, is 10^9-10^{10} less than the maximum substrate consumption rate (Table 2), which

would seem to indicate that as yet unidentified factors play a role in restraining the microbial respiration.

The hot, reducing, gaseous water emanating from a fracture at 2.8 to 4.2 kmbls harbored a microbial community dominated by a single *Firmicutes* phylotype. The *Firmicutes* probably penetrated the Mponeng fracture zone at current depths during infiltration of paleometeoric water between 3 and 25 Ma and since then have relied upon non-photosynthetically-derived H_2 and SO_4^{2-} converted from Archaean/Proterozoic pyrite/barite. Nutrient concentrations have remained much higher than observed in shallower crustal environments, suggesting that the deep crustal biosphere may be energy rich, is not approaching entropic death and is capable of sustaining microbial communities indefinitely by geological processes.

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Table 1. Geochemical and microbiological characteristics of fracture water and mining water.

Sample No. (number of days since intersection)	1 (4)	2 (7)	3 (15)	4 (58)	5
Sample name	MP104E65XC-091602	MP104E65XC-091902	MP104E65XC-092702	MP104E65XC-110902	MP104E65XC-SW-091602
Origin	Fracture water	Fracture water	Fracture water	Fracture water	Mining water
Water and gas flow rates (L min^{-1}) (borehole volumes)	40/2.4 (45)	10.9/0.8 (55)	8.2/1.2 (64)	2.3/1.7 (96)	NA
pH	9.3	9.3	9.3	9.2	NA
Eh (mV)	-330	-350	-340	-263	NA
Temperature (^oC)	>60	52	52	52	20
Formate (μM)	7.6	NA	7.1	8.9	1.4
Acetate (μM)	24.6	NA	22.5	35.7	5.9
Cl^- (mM)[1]	54.1	NA	71.9	84.9	0.42
Br^- (μM)	125	NA	177	218	3.8
SO_4^{2-} (μM)	529	NA	900	1860	171
HS^- (μM)[2]	NA	1390	NA	1060	NA
H_2 (μM)[2]	1940	2600	2090	3715	NA
CH_4 (μM)[2]	8580	11800	9320	16600	NA
O_2 (μM)[3]	6	<d.l.	<d.l.	<d.l.	285
δ^2H-H_2 (‰VSMOW)	-684	-684	-688	-695	NA
$\delta^{13}C$-CH_4 (‰PDB)	-31.6	-31.7	-32.8	-33.2	NA
δ^2H-CH_4 (‰VSMOW)	-364	-367	-366	-390	NA
^{4}He model age (Ma)	20.9±10.5	20.4±10.2	15.8±7.9	NA	NA
^{40}Ar model age (Ma)	16.3±8.2	21.3±10.6	16.9±8.4	NA	NA
^{134}Xe model age (Ma)	18.7±7.0	19.4±3.8	21.0±6.0	NA	NA
^{136}Xe model age (Ma)	21.6±6.0	25.0±3.8	23.8±4.6	NA	NA
Bacterial DNA (pg ml^{-1})	15±8	16±8	30±15	30±15	$3±1.5×10^5$
Archaeal DNA (pg ml^{-1})	~5E-4	<d.l.	~5E-4	~5E-4	206±100
Cell density (cells ml^{-1})	$5.1±0.5×10^4$	NA	NA	$3.3±0.3×10^4$	NA

[1]The concentrations derived from charged balance were 10.2 mM for sample #1, 27.6 mM for sample #3 and 50.6 mM for sample #4, respectively.

[2]The concentrations of dissolved gases were reported as concentrations corrected for diffusive loss (*8*). Diffusive correction was not applied to sample #4 due to the lack of noble gas analysis.

[3]The positive O_2 content for sample #1 may be derived from incomplete isolation of fracture water from the mining environment caused by the extremely high water pressure and flow rate.

[4]Carbon isotopic value was referenced to Pee Dee Belemnite (PDB).

[5]The uncertainties for aqueous and gas chemistry are ±10% and ±20%, respectively. The uncertainties for isotopic measurements are ±0.5‰ for $\delta^{13}C$-hydrocarbon and ±5‰ for δ^2H-hydrocarbon and δ^2H-H_2, respectively.

[6]NA: not available; <d.l.: below detection limit, which is 1 μM for O_2 and 0.5 fg ml^{-1} for archaeal DNA.

Table 2. Gibbs free energy, substrate consumption rate[1] and steady state free energy flux[1], FEF, for various microbial redox reactions.

Sample No.	1			3			4		
	ΔG kJ $mole^{-1}$	Substrate rate μM yr^{-1}	Energy flux kJ $cell^{-1}$ sec^{-1}	ΔG kJ $mole^{-1}$	Substrate rate μM yr^{-1}	Energy flux kJ $cell^{-1}$ sec^{-1}	ΔG kJ $mole^{-1}$	Substrate rate μM yr^{-1}	Energy flux kJ $cell^{-1}$ sec^{-1}
H_2-sulfate reduction	-148	5.9E+06	-9.2E-13	-155	8.6E+06	-1.4E-12	-146	1.8E+07	-2.7E-12
Acetate-sulfate reduction	-86	1.9E+05	-1.7E-14	-94	1.5E+05	-1.5E-14	-82	2.4E+05	-2.1E-14
H_2-methanogenesis	-94	2.1E+05	-2.1E-14	-95	2.9E+04	-2.9E-15	-93	3.8E+05	-3.7E-14
Acetate-methanogenesis	-33	1.9E+05	-6.6E-15	-34	1.5E+05	-5.5E-15	-28	2.4E+05	-7.1E-15
Formate-methanogenesis	-74	1.8E+04	-1.4E-15	-86	1.5E+04	-1.3E-15	-42	1.8E+03	-8.1E-17
H_2-Acetogenesis	-62	1.4E+05	-9.3E-15	-61	2.0E+04	-1.2E-15	-64	2.5E+05	-1.7E-14

[1]The calculation for maximum substrate consumption rate and steady state free energy flux is shown in SOM.

[2]The HS^- concentration used in the calculation for sulfate reduction for samples #1 and #3 were assumed to be 1.3 mM. The free energy for sulfate reduction only varied less than 5% when HS^- concentration was changed between 1.1 to 1.5 mM.

[3]The reactions for free energy calculations:

H_2-sulfate reduction: $4\ H_2 + H^+ + SO_4^{2-} \rightarrow HS^- + 4\ H_2O$

Acetate-sulfate reduction: $CH_3COO^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-$

H_2-methanogenesis: $4\ H_2 + H^+ + HCO_3^- \rightarrow CH_4 + 3\ H_2O$

Acetate-methanogenesis: $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$

Formate-methanogenesis: $4\ HCOO^- + H^+ + H_2O \rightarrow CH_4 + 3\ HCO_3^-$

H_2-Acetogenesis: $4\ H_2 + H^+ + 2\ HCO_3^- \rightarrow CH_3COO^- + 4\ H_2O$

[4]The uncertainties are ±10%.

Figure legends

Figure 1. Phylogenetic trees for bacteria (a) and archaea (b) based on 16S rDNA sequences recovered from fracture and mining water in the Mponeng Au mine, South Africa. The fraction number in the parenthesis represents the number of clones for each phylotype versus the total number of clones analyzed in each sample. The bar is equivalent to the sequence variation of 10%.