Chapter 6
Microbiology of the Deep Continental Biosphere

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Abstract Subsurface microbial communities in sediment and fractured rock environments beneath continental surface environments and beneath the ocean floor comprise a significant but largely unexplored portion of the Earth's biosphere. The continental subsurface is highly geologically varied, and so the abundance, diversity, and metabolic functions of its inhabitant microbes are even more widely ranging than those of marine systems. Microbial ecosystems in relatively shallow groundwater systems are largely fueled by organic carbon derived from photosynthesis, whereas deeper groundwater ecosystems are fueled by molecular hydrogen, methane, and short-chain hydrocarbons ("geogas"), produced by abiotic water–rock interactions, e.g., serpentinization and radiolysis of water. The abundances of microbes generally decline with depth, with deep fracture waters containing ~10^3–10^4 cells ml^{-1}; many of these microbes are metabolically active, albeit at very slow rates The depth limit of the biosphere may be controlled by a combination of temperature and other factors such as energy availability and pressure. Diverse bacteria and archaea appear to be adapted for life under the extremes posed by subterranean conditions. Further research is needed to explore a wider range of subsurface continental geologic settings, to constrain the rates of microbial metabolism, and to understand mechanisms of evolution in the subsurface.

6.1 Introduction

This chapter focuses on our current understanding of and major outstanding research questions regarding indigenous microorganisms in deep groundwater environments underlying the Earth's continents. Together with marine sediments and crust, these habitats are termed the "subsurface." In the deep relatively inaccessible regions that harbor life, they can be termed the "deep biosphere" or even "dark life." The study of deep subsurface habitats and their inhabitant microbes is

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relatively young, but has matured such that we no longer present it in terms of novel glimpses of a shadowy world; the mass of accumulated data has made it easier to defend the findings of microbes as not merely contaminant artifacts but as truly indigenous microbes functioning in active subsurface ecosystems (Fredrickson and Balkwill 2006). The existence of the deep biosphere is now well entrenched in the textbooks (e.g., Madigan et al. 2013). Indeed, the portion of the biosphere comprising subsurface continental as well as marine environments is now recognized as containing a significant proportion of the Earth's microbial cells, possibly even the majority (Whitman et al. 1998; Kallmeyer et al. 2012; Røy et al. 2012). Early studies, primarily in the vicinities of petroleum reservoirs beginning in the 1920s (Bastin et al. 1926), set the stage for concerted programs, e.g., by the U.S. Environmental Protection Agency and the U.S. Department of Energy in the 1980s, which probed subsurface aquifers, at first to only a few meters (Wilson et al. 1983), but then followed by increasingly deep drilling to depths approaching 3 km (Onstott et al. 1998). In addition to drilling from the surface as a means of accessing the deep biosphere, microbiologists began descending into deep mines to sample groundwater via boreholes drilled from within the mines and extending outward into pristine, often ancient groundwater (Kieft et al. 1999; Onstott et al. 2003). Both of these approaches are now widely used and are unearthing new discoveries on a regular basis. There is now a history of reviews on the topic (e.g., Fredrickson and Onstott 1996; Amy and Haldeman 1997; Chapelle 2000; Pedersen 2000; Amend and Teske 2005; Onstott et al. 2009; Colwell and D'Hondt 2013), which serve as a background for this chapter. While there are many practical motivations for studying subsurface microbiology (e.g., understanding contaminant fate and transport, developing hazardous waste repositories, etc.), this review focuses on recent findings and prospects in the basic science of mostly uncontaminated continental subsurface environments.

Much of our current understanding of the microbiology of groundwater was succinctly summarized by T.C. Onstott in a diagram created for the National Science Foundation's EarthLab report (NSF 2003) (Fig. 6.1). Energy sources in shallow subsurface environs are seen to be primarily photosynthetically generated organic carbon that can occur as detritus buried in sediments and as organic matter transported via diffusion and advection in groundwater to depth. The quantity and quality of this organic carbon available for microbial metabolism decline with depth, and so the abundance of the dominantly heterotrophic bacteria in these aquifers also declines with depth. The general patterns of abundance and biogeochemical activities of microbes in these relatively shallow aquifers were reasonably well characterized 20 years ago; regional groundwater flow results in a predictable sequence of terminal electron-accepting processes along a flow path, from aerobic metabolism near the recharge zone to anaerobic processes such as methanogenesis in the most distal regions (Murphy et al. 1992; Lovley et al. 1994) (Fig. 6.2). Also, microbial metabolism of buried organic matter in fine-textured aquitards results in diffusion of fermentation products to adjacent sandy aquifers, where they serve as electron donors stimulating microbial activities at boundaries between layers. Rates of activity generally decline as one proceeds from surface environments to more

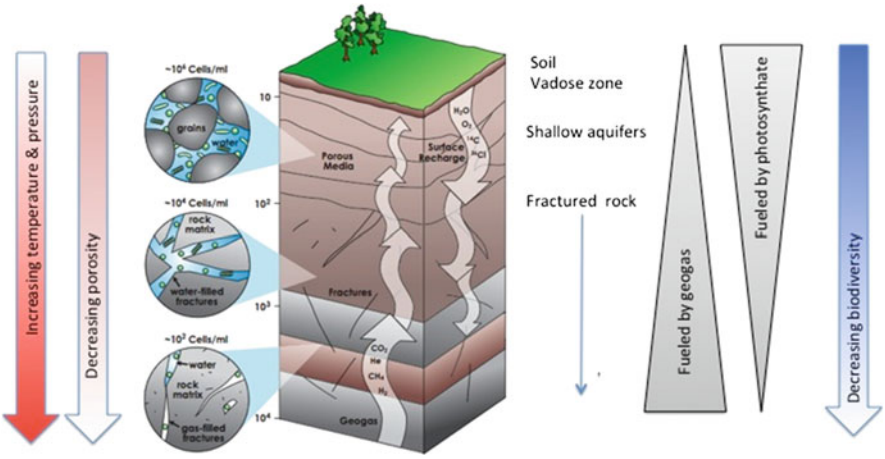

Fig. 6.1 Overview of subsurface continental environments. Microbial abundance in pore water generally declines with depth, from shallow aquifers to underlying fractured rock environments. The quantity and quality of photosynthetically derived organic C decline with depth, and the importance of "geogas" energy sources (e.g., H_2, CH_4) generally increases with depth. Temperature increases with depth according to the local geothermal gradient; pressure increases with depth (~10 MPa/km). (modified from EarthLab, National Science Foundation 2003)

static subsurface systems (Phelps et al. 1994; Kieft and Phelps 1997) (Fig. 6.3). Proceeding further into the subsurface (Fig. 6.1), one encounters the bedrock underlying surface sediments and the porosity generally diminishes with depth, as well. As the availability of photosynthate declines with depth, microbial abundance declines, and H_2 generated abiotically through water–rock interactions, termed "geogas" by Pedersen (1997), is the dominant energy source in the deepest regions of the biosphere (Fig. 6.4). Some of the most exciting current work in the subsurface, both continental and marine, involves determining the rates and extent of these H_2-fueled ecosystems and characterizing the communities therein.

Although the field has matured, there are still many outstanding research questions. Broad research questions include the following, as organized by an U.S. National Science Foundation-sponsored group (Fredrickson et al. 2006):

- How deeply does life extend into the Earth?
- What fuels the deep biosphere?
- How does the interplay between biology and geology shape the subsurface?
- What are subsurface genomes telling us?
- Did life on the earth's surface originate underground?
- Is there life in the subsurface as we don't know it?

More specific questions that are being or should be addressed include:

- What is the physiological state of subsurface microbes, i.e., are they slowly metabolizing, "healthy" cells that are well adapted to the rigors of subsurface life, or are they ill-adapted, moribund cells in slow decline?

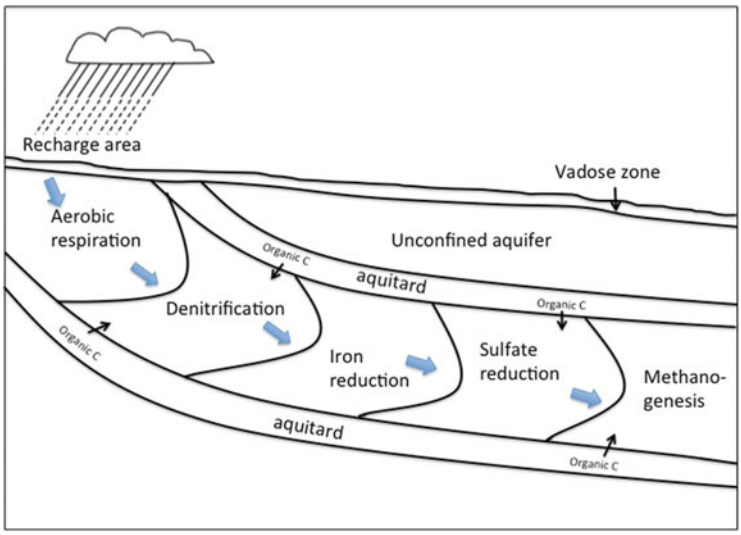

Fig. 6.2 Idealized sequence of terminal electron-accepting processes occurring along a flow path over tens of km in a confined aquifer. Buried organic carbon in confining aquitards is slowly degraded and fermentation end products diffuse into the aquifer, creating zones of high microbial activity at the aquitard–aquifer interfaces. (After Smith and Harris 2007)

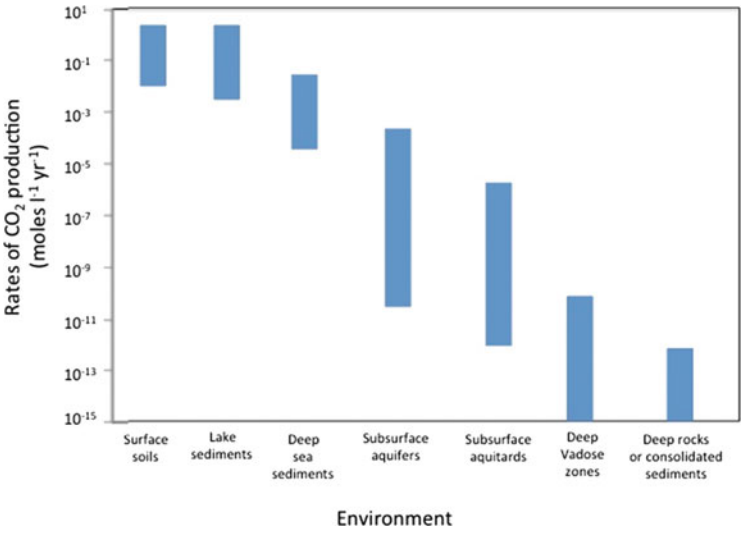

Fig. 6.3 Ranges of rates of in situ CO_2 production for various environments. Subsurface rates were estimated from groundwater chemical analyses and geochemical modeling. (After Kieft and Phelps 1997)

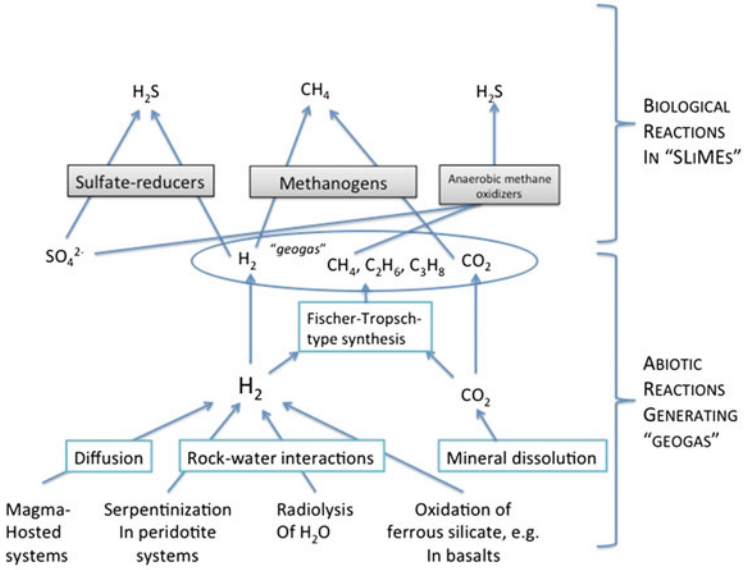

Fig. 6.4 Abiotic subsurface processes that generate H_2, CH_4, and CO_2 ("geogas") and example subsurface lithoautotrophic ecosystems ("SLiMEs") that are fueled by geogas

- By what means and at what rates are microbes transported to the subsurface and within subsurface environments?
- What's the role of lateral gene transfer among subsurface populations?
- How do the planktonic cells sampled from the bulk water phase differ from sessile, biofilm communities?
- What are the interactions within subsurface microbial communities?

6.2 Comparison of Deep Continental and Marine Biospheres

While continental and marine subsurface environments have much in common (dark anaerobic ecosystems, slow metabolic rates, heterotrophic metabolism of buried and transported organic C, and evidence for chemolithoautotrophic ecosystems), the study of the two has remained largely separate (Colwell and Smith 2004). We now know a great deal about the marine deep biosphere—both deep hot (vent related) subsurface marine systems (Huber et al. 2007; Kelley et al. 2005; Wang et al. 2009) and not so hot subseafloor sediments (D'Hondt et al. 2004; Kallmeyer et al. 2012). However, the vent and vent-related systems are essentially localized, hot, chemoautotrophic systems and the sediments are vast, cold heterotrophic systems functioning on old, buried photosynthate. The deep continental biosphere in contrast contains what could be termed "cool" chemoautotrophic ecosystems,

cool in the geochemical context meaning anything <100 °C. The rock–water interactions that fuel these subsurface ecosystems are not dependent upon localized magmatic input, e.g., spreading centers, volcanic activity, or hot spots, but instead are influenced by regional, topographically driven fluid flow, e.g., in the cases of the elevated plateau containing the Witwatersrand Basin in South Africa (Gihring et al. 2006; Onstott et al. 2006), the "cooler," low elevation subsurface in granitic aquifers of the Fennoscandian Precambrian shield (Pedersen 1997, 2000; Itavaara et al. 2011), and Columbia River basaltic aquifers (Stevens and McKinley 1995, 2000).

6.3 Methods for Sampling the Subsurface

The deep biosphere is challenging to access, generally requiring drilling, either from the surface or from a preexisting subsurface site, e.g., in deep mines. Drilling is inherently messy, usually involving drilling fluids with potential for chemical and microbiological contamination. Techniques for minimizing contamination and for tracing and quantifying contaminant fluids and particulates were devised during the U.S. Department of Energy's Subsurface Science Program (Phelps et al. 1989; Colwell et al. 1992; Russell et al. 1992), and these have been adapted for use elsewhere, including the Integrated Ocean Drilling Program (Smith et al. 2000). Subsurface sampling approaches have been reviewed by Moser et al. (2001), Kieft et al. (2007), and Kieft (2010).

Drilling and coring to depths greater than ~300 m require rotary drilling using a drilling fluid, either liquid or gas, to lubricate and cool the drill bit and to remove the cuttings. These fluids can be problematic, especially when drilling muds with organic additives are used, because they favor the growth of contaminating microbes. Air or an inert gas, e.g., Ar, can be used and these can be filtered, although this requires a massive filter (Colwell et al. 1992). Water can be used, but denser fluids are generally required for very deep drilling. If possible, organic additives and petroleum-based lubricants should be avoided. Online gas analyses of the drilling fluid are commonly used in the oil and gas industry and can be employed in scientific drilling to identify biologically active zones (Erzinger et al. 2006). When cores are to be used for microbiological analyses, the usual approach is to deploy solute and particulate tracers. Solute tracers added to the drilling fluid include fluorescent dyes, LiBr, and perfluorinated hydrocarbons. The latter can be quantified over a broad concentration range by gas chromatography. Particulate tracers include microbe-sized (0.5 or 1.0 μm diameter) fluorescent microbeads that are carboxylated to mimic the negative surface charge on most bacteria. These are added in a plastic bag at the bottom of the core barrel such that the bag is broken on contact with the formation and the beads are mixed with fluid that contacts the core. A subcore is removed from the interior of the core and tracers are quantified in the parings from the core perimeter and in the subcore. Ideally, the subcore should have ≥10,000-fold lower concentration of tracers than the parings.

Microbial communities in the drilling fluid and in the subcore can also be compared as a further test for drilling-induced contamination (Lehman et al. 1995; Dong et al. 2014). Sidewall coring is another option for collecting solids (Colwell et al. 1997; Dong et al. 2014), but the volume is very limited. Samples are known to change their microbiological composition very shortly after collection, so they should be processed as soon as possible (Brockman et al. 1998). Samples intended for analysis of nucleic acids, proteins, lipids, etc., should be frozen immediately, if possible. Samples intended for cultivation of microbes should be handled in a glove bag containing an inert atmosphere to preserve oxygen-sensitive anaerobes.

Following drilling, boreholes can be sampled for groundwater as long as the borehole is flushed sufficiently to remove contaminating solutes and microbes in the open borehole. Discrete depth intervals can be targeted using packers (Dong et al. 2014). Multilevel samplers can sample from more than one depth interval. A U-tube system (Freifeld 2009) can also be used for collecting from depth. Microbes suspended in groundwater can be collected and concentrated onto filters (Moser et al. 2005; Gihring et al. 2006). Wireline formation testers can monitor borehole water chemistry and also sample water from central and outer portions of the rock formation before coring (Dong et al. 2014). Devices can also be inserted into the borehole for long-term monitoring and collection of samples and to enrich for subsurface microbes (Orcutt et al. 2010, 2011: Silver et al. 2010).

Drilling from existing deep underground sites, e.g., from within mines, confers the advantages of lower costs and also facilitates drilling to great depth without having to begin with a wide diameter collar. This approach has enabled collection of some of the deepest fluids so far collected from the subsurface (Moser et al. 2003, 2005; Kieft et al. 2005; Borgonie et al. 2011; Lippmann-Pipke et al. 2011b). Boreholes, drilled into surrounding pristine rock, generally as part of mine operations, can intersect ancient fracture water that then flows from the borehole, flushing drilling contaminants and carrying inhabitant microbes that can be collected by filtration. Sterile packers connected to a manifold system can be used to transfer water and gas samples for filters, sample vials, etc., without exposure to mine air (Fig. 6.5). Unfortunately, mining interests rarely overlap with the interests of geomicrobiologists, so opportunities for sampling in mines tend to be few and short lived. For this reason, sampling and long-term monitoring and experimentation in dedicated underground laboratories are an attractive option (Pedersen 1997, 2000; Edwards et al. 2006; Onstott et al. 2009; Fukuda et al. 2010).

6.4 Microbial Abundance

One of the simplest questions to ask is "How many are there?" Shallow sedimentary aquifers may harbor nearly 10^7 cells ml^{-1} of groundwater (Sinclair and Ghiorse 1989), nearly all of these being prokaryotic (bacteria and archaea). Microbial abundance generally declines with depth (Onstott et al. 1999; Itavaara et al. 2011), and the abundance of cells in fracture water collected from deep

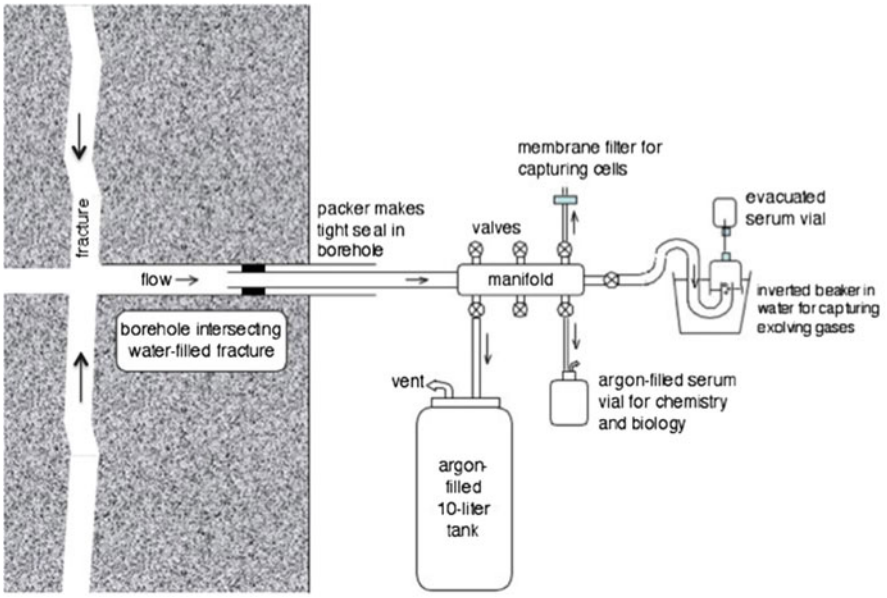

Fig. 6.5 Packer-manifold system for collecting deep fracture waters from boreholes drilled in mines. The borehole intersects a fracture filled with pressurized fluid. The flowing fracture water flushes the borehole of contaminants. The sterilized packer and manifold allow collection of water and gases without contamination from mine air. (Modified from Kieft et al. 2007, with permission from ASM Press)

crystalline rock is markedly lower than in sedimentary aquifers. Abundance of microbes in continental subsurface environments appears to decline more slowly than in the pattern described by Parkes for marine sediments (Onstott et al. 1999; Parkes et al. 1994). Flow cytometric counts of cells in deep fracture water collected from boreholes at depths of ~2–3.5 km in mines from across the Witwatersrand Basin in South Africa ranged from 2.5×10^2 to 5.9×10^4 cells ml^{-1} (Onstott et al. 2006). A more recent paper reports 9.0×10^1 to 2.2×10^3 cells ml^{-1} in fracture water at 1.8 km depth (Davidson et al. 2011). Pedersen (1997) reported 1.2×10^4 to 9.2×10^4 bacteria ml^{-1} in granitic fracture water at 450 m depth in the Äspö Hard Rock Lab in Sweden. Itavaara et al. (2011) published cell numbers declining from ~4.6×10^5 at 100 m depth to ~6×10^4 at 1500 m in crystalline rock aquifers in Finland. Fukuda et al. (2010) counted 1.1×10^4 to 5.2×10^4 cells ml^{-1} in ~1.1-km depth fracture water accessed in the Mizunami Underground Research Laboratory in Japan. These low numbers of subsurface microbes reflect the sluggish energy fluxes and possibly other limiting factors, and they make further microbial characterization especially challenging.

6.5 What Controls the Depth Limit of the Biosphere?

The deepest limit of the continental biosphere has yet to be clearly delineated at any site and so the factors that control that lower depth limit are not well understood. Temperature is the least forgiving of environmental parameters and therefore a first approximation of the deepest extent of the biosphere can be set at the ~121 °C isotherm. Reports have pushed the upper temperature for microbial proliferation to 121 °C (Kashefi and Lovley 2003) and even 122 °C when combined with elevated pressure (Takai et al. 2008), and the record may even be broken again, but most investigators would likely agree that it won't be by much. Combining this upper temperature limit for life with geothermal gradients that range from ~8 to 30 °C/km, one can estimate the deep limit of life to range from 2 to 12 km below land surface (kmbls) for mean surface temperatures from 0 to 25 °C. The Witwatersrand Basin of South Africa is a stable cratonic region where the geothermal gradient is low at 8–10 °C km^{-1} (Omar et al. 2003), so the biosphere could theoretically exceed 10 km, more than twice as deep and hot as has been probed thus far. A hole drilled in the Songliao Basin in China and cored for microbiological analysis may actually have probed beyond the lower limit of the biosphere (Dong 2009). A sharp drop in microbial biosignatures at a depth corresponding to ~120 °C supports temperature as the ultimate arbiter of microbial distribution. However, evidence from other sites suggests that factors besides temperature may also be at work; these include pressure, availability of pore space, energy flux, and inorganic nutrient availability.

Patterns of biodegradation of petroleum hydrocarbons with depth and temperature within reservoirs have been invoked to suggest an upper limit for microbial activities of ~80 °C (Wilhelms et al. 2001; Head et al. 2003). Petroleum reservoirs commonly harbor indigenous microbes, and in fact, the first cultivation of subsurface microbes, in this case, sulfate reducers, was from a petroleum reservoir (Bastin et al. 1926). Evidence of petroleum biodegradation includes absence of low-molecular-weight constituents and a preponderance of heavy oil, concentration of metals such as Va and Ni, accumulation of biogenic methane, and accumulation of isotopically heavy CO_2; these signatures of biological degradation are common in reservoirs where the temperature is below 80 °C, but appear not to occur at warmer temperatures. Head et al. (2003) attribute the lack of biodegradation at elevated temperatures to such factors as low fluxes of electron acceptors and inorganic nutrients and concomitant rates of metabolism that are so slow as to be unable to keep pace with the thermal breakdown of cellular constituents. Indeed, the currently described bacteria and archaea functioning at temperatures exceeding 80 °C are all from thermal springs and deep sea hydrothermal vents, where high concentrations of readily metabolized substrates (e.g., H_2, H_2S) interface with favorable electron acceptors, e.g., O_2. Slow energy fluxes are likely the rule in deep subsurface habitats other than petroleum reservoirs, as well, especially those in which electron donor generation is exclusively via rock–water interactions. The maintenance demands of life at temperatures exceeding 80 °C may simply be too great for the "slow-lane" lifestyle of subsurface microbes.

Onstott et al. (2014) reported that the combination of energy limitation and high temperature can limit microbes in subsurface environments due to the racemization of amino acids, specifically aspartate. Amino acids spontaneously racemize at higher rates with increasing temperature and aspartate racemizes more rapidly than other amino acids. This racemization requires replacement of proteins, which may demand more energy than is available in most subsurface environments with elevated temperature. Protein turnover times, estimated from amino acid D/L ratios, were found to be shorter in deep groundwater than expected: ~27 years at 1 km depth and 27 °C and 1–2 years at 3 km and 54 °C in the Witwatersrand Basin, South Africa. Amino acid racemization may be a factor explaining the ~80–90 °C limit to petroleum biodegradation in deep, hot petroleum reservoirs (Wilhelms et al. 2001; Head et al. 2003).

Although the pressures at depth in the continental subsurface are less than those in the deepest regions of the marine environment, they may nonetheless influence microbial activities. For example, the combined effect of hydrostatic and geostatic pressures at 3 kmbls where studies have been focused in South Africa can be estimated at ~30 Mpa, which is nowhere near the extreme pressures found in the ocean's deepest trenches, ~100 MPA. Nonetheless, these deep continental groundwater pressures may be sufficient to negatively impact growth rates, especially when combined with elevated temperature. Alternatively, in situ pressures may select for piezophilic, continental fracture water microbes, as has been shown for deep marine habitats (Bartlett. 2009). If obligate barophiles exist, then our current efforts at cultivation are missing them, as collection and incubation under pressure have so far not been conducted. There's a definite need for such studies in deep continental environs.

The effects of high pressure have been well studied in deep marine environments (Yayanos 1995, 2001; DeLong et al. 1997; Lauro and Bartlett 2008; Nagata et al. 2010); however, relatively little is known of the responses and adaptations of deep terrestrial microorganisms to high temperature. To date, there have been no published studies of the responses of deep subsurface terrestrial microorganisms to high pressure. While many of the responses are likely similar to those of marine microbes, the other in situ parameters associated with high pressure in the deep terrestrial biosphere can be very different. Temperatures in the deep ocean are cold (away from spreading centers), whereas the deep continental realm is warm (Yayanos 1995; Nagata et al. 2010). Moreover, high pressure in the deep Earth can be accompanied by very high partial pressures of various dissolved gases, e.g., H_2, CH_4. These can serve as energy sources, but may also affect metabolic processes in other ways. Piezophilic marine microbes are now being better characterized by high-throughput metagenomic and metatranscriptomic sequencing (Eloe et al. 2011; Wu et al. 2013). Similar approaches that are ongoing with deep continental groundwater samples may reveal similar or contrasting responses to elevated pressure.

Although porosity generally declines with depth, water-filled fractures do exist even at extreme depths (Stober and Bucher 2004), although the tortuosity of the fluid phase may be so great as to preclude transport of microbes. Some fracture

fluids may even be totally sequestered from the biosphere, with no opportunity for colonization by microbes. In some cases, deep fractures may contain ancient water that has been geohydrologically sequestered from other groundwater for millions of years (Lippmann-Pipke et al. 2003), although these are bulk ages and mixing with younger, paleometeoric water appears to be common. Microbes that persist in such ancient, sequestered waters must either survive on endogenous energy reserves or metabolize exogenous substrates that become biologically available in these environments. A flux of exogenous, energy-rich substrates, even if sluggish or sporadic, is required for truly long-term persistence.

6.6 Geogas and SLiMES

While the majority of subsurface ecosystems studied to date are powered by organic carbon derived from photosynthesis at the surface, exciting studies have revealed chemosynthetic subterranean ecosystems (termed SLiMEs, subsurface lithoautotrophic microbial ecosystems) that gain energy from geochemically generated inorganic energy sources, e.g., H_2 (Pedersen 1993, 1997; Stevens and McKinley 1995; Chapelle et al. 2002; Nealson et al. 2005). Thomas Gold (1992) first speculated on a vast subsurface, chemolithoautotrophically driven "deep, hot biosphere," and subsequent field studies have clearly demonstrated these ecosystems in isolated locations such as the groundwater feeding Liddy hot springs in Idaho (Chapelle et al. 2002), the Lost City vents in the Atlantic Ocean (Kelley et al. 2005), and deep fracture waters in the Witwatersrand, South Africa (Lin et al. 2006; Chivian et al. 2008). As these are generally anaerobic systems, they're totally independent of the products of photosynthesis, both O_2 and organic carbon, unlike deep sea hydrothermal vent ecosystems. Abiotic rock–water interactions that generate H_2 include serpentinization of ultramafic rocks (Schrenk et al. 2013), oxidation of ferrous silicate minerals in basaltic aquifers (Stevens and McKinley 2000), and radiolysis of water in environments with significant radiation flux (Lin et al. 2006). The H_2 in turn generates CH_4 and short-chain hydrocarbons via Fischer–Tropsch type synthesis reactions (Sherwood Lollar et al. 2002, 2007). Carbon monoxide is also commonly found in deep subsurface waters and is thought to be geochemical in origin (Kieft et al. 2005; Gihring et al. 2006; Onstott et al. 2006). Together, the H_2, CH_4, short-chain hydrocarbons, and CO (geogas) make for relatively energetic anaerobic ecosystems that appear to be dominated by a few species of prokaryotes, including methanogens and sulfate reducers. Considering the widespread occurrences of basaltic crust, serpentinizing low-silicate ultramafic rocks, and sources of gamma irradiation in the subsurface, SLiMEs may underlie large areas of the Earth, possibly approaching the ubiquity (but not the extreme depth) posited by Gold (1992).

High concentrations of H_2 have been measured in Precambrian Shield groundwaters in Canada, South Africa, and Finland, with values as high as 7.4 mM in the Witwatersrand Basin (Sherwood Lollar et al. 2007). The question then arises as to

how such high concentrations of an easily metabolized energy source can accumulate without being oxidized by microorganisms. These high concentrations are found in the deepest, most saline fracture waters, with bulk water ages of millions to tens of millions of years (Lippmann-Pipke et al. 2003). While limitation by inorganic nutrients may curb microbial activity in some cases (Kieft et al. 2005), Sherwood Lollar et al. (2007) suggested that ancient H_2-rich saline waters are hydrogeologically isolated and are released to mix with other waters only sporadically, possibly due to widening of pore-throat diameters following tectonic shifts. Lippmann-Pipke et al. (2011a) simultaneously monitored mining-induced seismicity (blasting) and geogas concentrations, including H_2 and CH_4, at 3.54 km depth in TauTona mine in South Africa, and found that spikes in geogas concentrations coincided with the daily blasting schedule in the mine. This finding has important implications for the metabolism of geogas by SLiMEs in fractured rock.

6.7 Subsurface Biodiversity

Microbial communities in deep subsurface continental habitats can be diverse and they vary with geochemical conditions (Gihring et al. 2006). Many of the microbes detected in culture-independent surveys represent novel lineages (Takai et al. 2001a; Gihring et al. 2006; Chivian et al. 2008; Sahl et al. 2008), many of which appear to be unique to subsurface environs. Some of these are cosmopolitan in their distribution, being detected as very similar small subunit rRNA gene sequences in water from boreholes that are widely separated geographically, even on different continents. The Firmicute *Candidatus Desulforudis audaxviator* is a case in point, having been detected in borehole waters from across the Witwatersrand Basin in South Africa (Moser et al. 2005; Lin et al. 2006; Chivian et al. 2008), as well as in Finland (Itavaara et al. 2011), and beneath Death Valley in California (Moser 2012). Other sequences have been detected in only a single borehole, suggesting that these represent microbes that are uniquely adapted to conditions at that site or that they're part of the so-called "rare biosphere" (Sogin et al. 2006). Surveys of microbial diversity in deep subsurface environs frequently reveal novel taxa, including previously unknown microbial phyla and phyla with no known cultivated representatives (Gihring et al. 2006; Chivian et al. 2008; Sahl et al. 2008; Dong et al. 2014). In one case, a previously unknown prokaryotic cell morphology, rod-shaped cells that are five-pointed and six-pointed stars in cross section, was discovered in a deep platinum mine in South Africa (Wanger et al. 2008). Clearly the deep continental biosphere is expanding our understanding of biodiversity.

A general pattern of decreasing diversity with depth has been observed (Gihring et al. 2006; Lin et al. 2012a). Explanations for the decreasing biodiversity include the extreme physical-chemical conditions (alkaline pH, elevated temperature, hydrostatic pressure), the limited number of available substrates, and the limited opportunities for colonization by immigrant microbes (Gihring et al. 2006). The

ultimate in low diversity may be found in the simple, one-species ecosystem reported by Chivian for planktonic microbes collected from fracture water at 2.8 km depth in Mponeng gold mine in South Africa (Lin et al. 2006; Chivian et al. 2008). The dominance (95 % of the community) of hydrogenotrophic methanogens in the groundwater feeding Lidy Hot Springs in Idaho (Chapelle et al. 2002) may be another example. In both of these cases, chemolithotrophs are metabolizing H_2 generated by abiotic water–rock interactions, without the need for fermentation or syntrophs.

Archaea are commonly detected in deep continental fracture waters (Takai et al. 2001a; Moser et al. 2005; Gihring et al. 2006; Davidson et al. 2011; Nyssönen et al. 2012). Takai et al. (2001a) detected novel groups of Euryarchaeota (SAGMEG 1 and 2) and Crenarchaeota SAGMCG 1 and 2). The Crenarchaeota may be primarily drilling fluid contaminants, whereas the methanogenic Euryarchaeota may be indigenous groundwater archaea (Gihring et al. 2006; Davidson et al. 2011). So far, we have hints of anaerobic methane-oxidizing archaea (ANME) in the deep continental surface (Gihring et al. 2006) in the form of ANME-related 16S rDNA sequences, but they appear not to be dominant, and the energetics of anaerobic methane oxidation coupled to sulfate reduction appears to be overshadowed by other more favorable reactions (Moser et al. 2005; Kieft et al. 2005).

Sequences representing diverse bacterial phyla have been detected in the subsurface, with Proteobacteria and Firmicutes often predominating (Gihring et al. 2006; Moser et al. 2003; Itavaara et al. 2011). Alpha-, beta-, and gammaproteobacteria are commonly found. In some cases, these appear to comprise a greater proportion of the community when water is first sampled from a borehole, but then later diminish as the borehole community becomes dominated by indigenous fracture water microbes (Sahl et al. 2008; Moser et al. 2003; Davidson et al. 2011). In other cases, the Proteobacteria appear to truly dominate (Dong et al. 2014). Sulfate-reducing bacteria of the deltaproteobacteria are also reported (Onstott et al. 2003; Itavaara et al. 2011; Davidson et al. 2011; Nyssönen et al. 2012). Proteobacteria may be more likely to dominate in shallower aquifers (Itavaara et al. 2011; Lin et al. 2012a). Firmicute sequences frequently encountered include sulfate-reducing, spore-forming genera, e.g., *Cand. D. audaxviator* and *Desulfotomaculum* spp., as well as *Thermoanaerobacter* spp. (Gihring et al. 2006; Davidson et al. 2011; Nyssönen et al. 2012; Aüllo et al. 2013).

The sulfate-reducing Firmicute *Cand. D. audaxviator* deserves special attention. Culture-independent surveys of deep fracture water communities in the Witwatersrand Basin, South Africa, have repeatedly found closely related ($\geq$99 % homology) 16S rRNA sequences of a novel Firmicute with the most closely related cultured organisms belonging to the genus *Desulfotomacum* (Baker et al. 2003; Moser et al. 2003, 2005; Gihring et al. 2006). At 2.8 km depth in fracture water accessed via a borehole in Mponeng mine, it was found to comprise >99 % of the community of suspended cells. All evidence indicates that this organism can function as a chemoautotrophic sulfate reducer in a simple ecosystem using radiolytically generated H_2 and sulfate chemically oxidized from pyrite by radiolytically generated

oxygen species. Genomic sequencing revealed hygrogenase, a complete dissimilatory sulfate reduction pathway, and capability for fixing CO_2 via the Wood–Ljungdahl pathway, thereby confirming its role in this simple ecosystem (Fig. 6.6) (Chivian et al. 2008). Other features revealed in the genome include genes for endospores, flagella, nitrogen fixation, and evidence for horizontal gene transfer from other bacteria as well as archaea, and it was described as the new candidate species *D. audaxviator*. Despite numerous attempts and knowledge of its full genome, no one has successfully isolated a member of this species (however, a related organism has now been grown in enrichment culture (Duane Moser, personal communication). Clearly, *D. audaxviator* is a highly successful organism, adapted to a wide range of subsurface physical and geochemical conditions. As described above, it's been detected in groundwater from three continents, but so far not detected in surface environments. A related sequence was found in deep marine crustal fluid (Cowen et al. 2003). Its ubiquity in widely spaced, hydrogeologically isolated, ancient fracture waters presents a biogeographical conundrum. Presumably, the genomes of the widely flung versions of this organism vary to a greater degree than is evident in their 16S rRNA genes. This hypothesis is being tested by Ramunas Stepauskas using the single cell genomics approach (personal communication). As such, the various versions of this species may be analogous to Darwin's finches, being among the few organisms to survive transport to remote deep environs and then adapting to the specific and varied conditions there, an idea put forth by Tullis Onstott and Ramunas Stepanauskas (personal communication).

The finding of large numbers of sequences from spore-forming Firmicutes raises the question of whether or not they're present as actively metabolizing vegetative cells or as inactive endospores. Arguments in favor of active cells include the observation by SEM of long, rod-shaped cells rather than spores in filtrate from the 2.8 km deep South African Mponeng mine water containing the simple, one-species *Cand. D. audaxviator* ecosystem (Chivian et al. 2008) and sulfur isotope fractionation in deep South African water samples that also have high proportions of sulfate-reducing Firmicute sequences (Lau et al. 2013). Evidence suggesting that the Firmicutes are present mainly as endospores lies in a comparison of DNA- and RNA-based surveys of microbial communities in deep South African fracture water communities. Several samples showed markedly higher proportions of Firmicute sequences in the DNA than in the RNA. This is a question that needs to be addressed further, e.g., using fluorescent in situ hybridization, analyses of mRNA as well as rRNA, stable isotope probing, and quantification of the endospore constituent calcium dipicolinate.

Eukarya are found only seldom in the deep continental biosphere and then generally not in the deepest, most anoxic groundwater. Sinclair and Ghiorse (1989) reported protozoa in relatively shallow (50–250 m) eastern coast plain aquifers of the United States. Lin et al. (2012b) reported a variety of protists in a shallow, unconfined aquifer at the Hanford Site, in Eastern Washington State, with a seasonal influence from the nearby Columbia River. Fungi were also reported by Sinclair and Ghiorse (1989) at 1–50 propagules per g dry weight of sediment; however, in deeper continental samples, fungi are generally not present and may

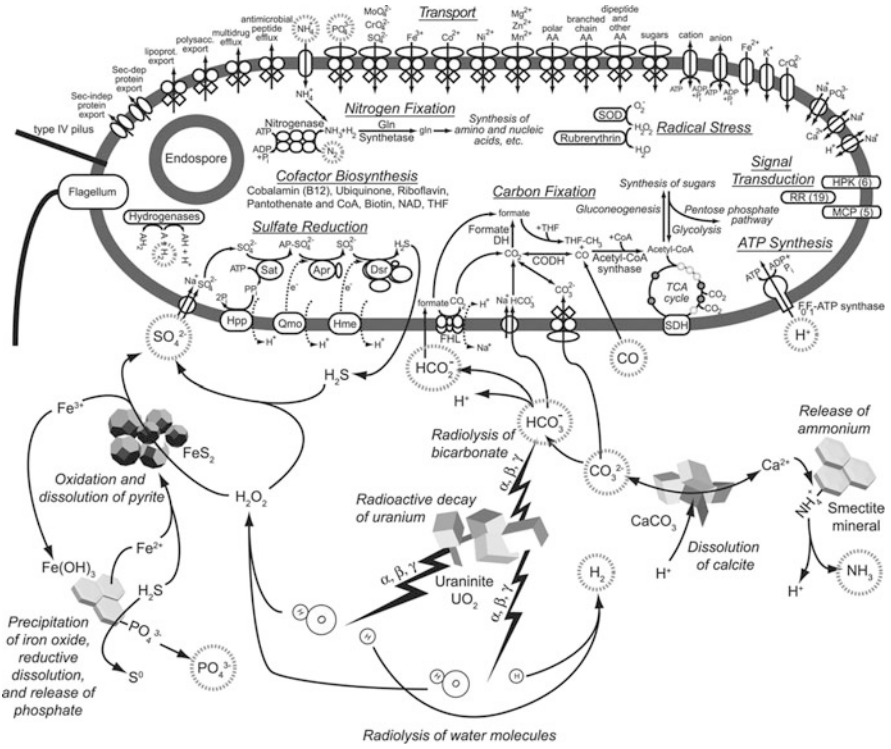

Fig. 6.6 Diagram of the genetically coded attributes of Candidatus *Desulforudis audaxviator* and its interactions with chemical components of its environment. (From Chivian et al. 2008, with permission)

even be considered to be serendipitous tracers of contamination (Onstott et al. 2003). Fungi have been reported in marine sediments (Orsi et al. 2013), so this may be another difference between continental and marine biospheres. Surprisingly, metazoan predators in the form of nematodes, including a novel genus, have been discovered feeding on bacteria in groundwater at depths of 0.9–3.6 km in the Witwatersrand Basin (Borgonie et al. 2011). These roundworms have been reported only from relatively young fracture waters (3000–12,000 years) containing low concentrations of dissolved O_2 (13–72 μM). Presumably, groundwater that's deeper, older, and anoxic does not contain metazoans.

Viruses occur in deep fracture water environments even though their host populations are relatively sparse. Kyle et al. (2008) reported 10^5–10^7 viral-like particles per ml of fracture water at the Äspö Hard Rock Laboratory (HRL) in Sweden at depths of 69–450 m, tenfold higher than the abundance of prokaryotes (10^4–10^6 cells ml^{-1}). Morphology indicated at least four different bacteriophage groups. Eydal et al. (2009) went on to isolate bacteriophages from these waters that specifically infected *Desulfovibrio aespoeensis*, which was previously isolated from these waters. Other evidence of subsurface bacteriophages is less direct. The

genome of *Cand. D. audaxviator* contains CRISPR-cas system genes (clustered regularly spaced short palindromic repeats, genomic sequences, which in combination with Cas proteins, serve as a prokaryotic acquired immune system important in resistance to foreign genetic elements such as plasmids and phages), possibly indicating past exposure to bacteriophages. Labonté et al. (2015) reported abundant temperate phage sequences in the genomes of subsurface bacteria. Viruses may be major controllers of subsurface bacterial and archaeal populations and they may also be important vectors for horizontal gene transfer among subsurface microbes (Anderson et al. 2013). Viruses in the subsurface deserve greater attention.

Diverse subsurface bacteria and archaea have been isolated in culture, although as in most environments, these are often not the important players in culture-independent, sequence-based surveys. The U.S. Department of Energy's Subsurface Science Program isolated thousands of strains of bacteria, mostly aerobic heterotrophs from subsurface samples (Balkwill and Boone 1997; Balkwill et al. 1997). Deeper sampling has produced primarily anaerobes (Table 6.1). Spore-forming Firmicutes, metal reducers, and iron reducers are well represented. Few methanogens have been isolated, likely due to sampling and cultivation challenges associated with their extreme O_2 sensitivity. Bonin and Boone (2004) cultivated a methanogen from a South African mine sample. These isolates can serve as useful model organisms that share metabolic characteristics with the more numerically dominant microbes. For example, a *Desulfotomaculum putei* from the Taylorsville Basin in Virginia (Liu et al. 1997) was used to quantify sulfur isotope fractionation ($\Delta^{34}S$) in a biomass-recycling turbidostat that modeled energy-limiting conditions in the subsurface; the magnitude of $\Delta^{34}S$ increased with energy limitation (Davidson et al. 2009). Many novel subsurface isolates have potential for biotechnological applications. Metal-reducing *Thermoanaerobacter* strains from deep sedimentary basins have been put to work synthesizing specialty minerals and immobilizing metal and radionuclide contaminants (Moon et al. 2007; Yeary et al. 2011; Madden et al. 2012). *Thermus scotoductus* strain SA-01 also has potential applications as a metal reducer (Opperman et al. 2010; Cason et al. 2012). Other cultures have been and will be isolated, with the potential for uses as model organisms and with potential for biotechnological applications.

6.8 Outlook for the Deep Continental Biosphere

The deep biosphere, both continental and marine, offers huge potential for discovery, and important revelations are made with each new opportunity to probe the subsurface. Nonetheless, the sobriquet "dark life" remains very appropriate. The volume of the subsurface that has been sampled is minuscule compared to the total volume of the deep continental biosphere, and more importantly, there remains a tremendous diversity of geological settings and habitat types that have yet to be examined. At present, the factor that is most limiting to progress in the continental subsurface is the ability to gain access to deep environments for sample collection,

Table 6.1 Selected deep subsurface microbial isolates. Selection was based upon the criteria of anaerobic growth or natural presence at depths >1 km

Isolate	Environment	Depth (km)	Characteristics	References
Bacillus infernus	Taylorsville Basin, Virginia sediment	2.7	Thermophilic (61 °C), strictly anaerobic, nitrate- and metal-reducing, endospore-forming Firmicute	Boone et al. (1995)
Desulfotomaculum putei	Taylorsville Basin, Virginia sediment	2.7	Thermophilic (50 °C) sulfate-reducing, endo-spore-forming Firmicute	Liu et al. (1997)
Desulfovibrio aespoeensis	Äspö Hard Rock Laboratory, Sweden	0.6	Sulfate-reducing deltaproteobacterium (25–30 °C)	Motamedi and Pedersen (1998)
Thermus scotoductus strain SA-01	Deep fracture water collected via Mponeng mine shaft 5, South Africa	3.2	Thermophile (65 °C), aerobic, and also nitrate and metal reducer	Kieft et al. (1999), Balkwill et al. (2004)
Alkaliphilus transvaalensis	Driefontein mine 5 shaft dam water	3.2	Thermophilic (20–50 °C,), alkaliphilic (pH 8–12.5) Firmicute; endospore former, strict anaerobe; heterotrophic nitrate, thiosulfate, and fumarate reducer	Takai et al. (2001b)
Thermoanaerobacter ethanolicus	Piceance Basin, Colorado	2.1	Thermophilic (60 °C) metal-reducing bacteria, produce magnetite	Roh et al. (2002)
Geobacillus thermoleovorans strain GE-7	Deep fracture water collected via Driefontein mine, South Africa	3.2	Thermophile (65 °C), aerobic, and also nitrate reducer	DeFlaun et al. (2007)
Halanaerocella petrolearia	Hypersaline oil reservoir, Gabon	1.15	Halophilic (growth at 6–126 % NaCl) fermen-tative Firmicute	Gales et al. (2011)
Desulfocurvus vexinensis	Deep aquifer, Paris Basin, France	0.83	Sulfate-reducing deltaproteobacterium (37 °C)	Klouche et al. (2009)

monitoring, and experimentation. While the necessary sampling equipment isn't quite as specialized and expensive as the drill ships used by the International Ocean Discovery Program, drilling on and in land is expensive, especially when the targets are >1 km deep. Drilling to significant depth generally requires millions of dollars and thus requires either major funding or the opportunity to piggyback a scientific investigation onto drilling carried out for other purposes, e.g., by the extractive industries and by entities seeking to dispose of hazardous waste (e.g., radionuclides,

CO_2). The International Scientific Drilling Program (ICDP) partially funds scientific drilling projects and has identified the deep biosphere as an important driver for future drilling expeditions (Harms et al. 2007), so it can be hoped that opportunities will expand. Drilling from underground platforms needs to be advanced via dedicated underground facilities, e.g., ones used for high-energy particle physics (Fredrickson et al. 2006; Onstott et al. 2009). Permanent facilities can be especially useful for long-term monitoring and experimentation.

While sampling opportunities have been limiting, the technologies for analyzing microbial communities and their metabolic activities have grown enormously. The entire "-omics" revolution (genomics, transcriptomics, proteomics, metabolomics) is being applied to the subsurface as well as nearly every other conceivable environment, and so one can expect the next decade to bring major breakthroughs. As we gain more and more data on the genetics and metabolism of subsurface microorganisms, the challenge will be to use these data to answer major questions such as how are the microbes interacting in situ, what are their rates of activity, and how did they get there and adapt to subsurface conditions in the first place? Better insight into Earth's dark life may also give insights to the consideration of life beneath the surfaces of other planets or even into the origin and early evolution of life on our own planet. There's no shortage of questions, and these days there's not even much limitation by available technology; we need only to expand the scientific will to drill.

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