What's up down there?

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The development of careful quality assurance criteria assuring freedom from contamination in all aspects of sample recovery has opened the window to studies of a fascinating new microbial biome in the deep subsurface. Organisms have been recovered with unusual metabolic capabilities and a chemosynthetic lifestyle independent of the recent surface photosynthetically derived energy inputs. The properties of the subsurface microbiota are critical when assessing aspects such as the utility of burying radioactive waste, the remediation of mixtures of organics, metals, and nuclides, and the search for life in extreme environments on Earth as well as on Mars and other extraterrestrial sites. In addition this pioneering work provides a foundation for examining life processes in extreme environments, such as the environment beneath the ocean floor.

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Abbreviation
PLFA phospholipid ester-linked fatty acids

Introduction

A general introduction to the history of the search for and the exploration of life within the deep subsurface, and an introduction to the interdisciplinary Deep Subsurface Science Program formulated by Frank J Wobber of the United States Department of Energy, was recently published [1••]. The Deep Subsurface Science Program, summarized in two recent publications [2•,3•], provided the scientific basis for current United States Department of Energy programs, such as The Natural and Accelerated Program in Bioremediation Research (NABIR), that utilize subsurface microbiota for bioremediation. In this review, we summarize the most recent significant advances in the understanding of 'what's up down there'.

Subsurface microbiota characterization

The evidence of microbial life in the deep subsurface has been postulated for a very long time, but the microbes that were recovered were suspected to be contaminants from the conventional biosphere. Interdisciplinary action by a team from the Department of Energy Deep Subsurface Science Program developed a set of criteria and methods by which sampling could be carefully controlled; tracers of various sorts, such as bromine ions, perfluorohydrocarbons, microbe-sized fluorescent beads, community biolog™ and signature lipid biomarkers, could be used to monitor contamination during the sample recovery process [4•]. One of the most powerful tests for contamination is community biology and phospholipid fatty acid signature analysis [5]. This shows distinct differences between microbial communities recovered from drilling muds, cuttings, and cores.

Subsurface community characterization

Phospholipid ester-linked fatty acids (PLFAs) are an excellent measure of the viable or potentially viable biomass in a wide range of environments. Viable microbes have intact membranes, which contain phospholipids (and PLFAs). Total PLFA often differs by three to five orders of magnitude between the drilling fluids and cores [5]. Cellular enzymes hydrolyze and release the phosphate group of phospholipids within minutes to hours following cell death [6]. The composition of the PLFAs and other lipids provides an insight into the community composition of the *in situ* microbiota [7•] and, because the lipids are modified in specific ways by shifts in the local environment, they reflect the physiological/nutritional status of the communities [8,9••]. The ratio of diglyceride fatty acids to PLFAs from lysed bacterial cells increases markedly with depth and the PLFA profiles can be significantly different at different subsurface horizons [10•]. Subsurface sediment samples collected in Western Washington, South Carolina, Northern New Mexico, and Central Idaho from a depth greater than 30 m were analyzed for PLFAs [11•]. Comparison of the PLFA profiles shows that the distribution of the microbial community is influenced by the geology of the subsurface: 78% of the variance in the community PLFA profiles is influenced by the lithology (sand/sandstone to clay/basalt) and an additional 7% by the permeability of the subsurface sedimentary element [11•]. The structure and chemistry of the subsurface clearly affect the microbial community composition and activity, but do the microbes affect the rocks? Rates of mineral weathering in aquifers have been related to bacterial colonization [12•]. Minerals that are not colonized by microbes are not weathered. Does soil mineral formation require a microbial biosphere?

The presence of dolomite (CaMg carbonate) in the geological record but not in present-day environments and the inability to generate dolomitic precipitates has been a long-standing enigma. It is possible that the 'dolomite problem' has a bacterial explanation. Vasconcelos and McKenzie [13•] showed that sulfate-reducing bacteria in hypersaline anoxic environments in nature and in laboratory microcosms can precipitate dolomite. Similar

environments in the subsurface could lead to dolomite precipitation.

Are the minerals precipitated by microbial action distinguishable from minerals formed by abiotic processes? A new finding may indicate that siderite (Fe-carbonate) formed by batch grown *Geobacter metallireducens* yields oxygen isotope values that are not in thermodynamic equilibrium with the oxygen isotopic composition of the medium [14•]. Possibly many biomineralization processes produce isotopic signatures that are distinct from what would be predicted from equilibrium thermodynamics. This could make possible the detection of fossil life processes in sedimentary rocks where microbes have not been detected.

Metabolic activity in the deep subsurface

The heterogeneous distribution of microbes in subsurface sediments was elegantly demonstrated by trapping ^{35}S sulfide on silver foil placed over a freshly fractured core face [15••]. Using this technique, it was possible to show high sulfide reduction activities in sandstones at the interfaces with shales. Nutrients leached from the shales probably support microbial communities at interfaces with sandstones. The shales have greatly decreased microbial activity because of the narrow pore throats restrict the access of the microbes to the nutrients [16••,17••].

The extant microbiota in the subsurface can be marvelously inactive in the harsh environment where life processes are very slow [16••]. Their diversity is great enough that they can respond quickly to nutrient if it appears. The disturbance artifact of exposure to nutrients at many orders of magnitude than found *in situ* when tested *in vitro* could possibly result in measurements of microbial activity more than four-orders of magnitude greater than their actual *in situ* activity. Beware of microbial activity determinations based on methods in which surface sediments are mixed vigorously with substrates in flasks—these are inappropriate for the subsurface environment. Even though nutrients may be present in the subsurface they may be localized in pores with throats too small for the microorganisms; therefore, diffusion may limit nutrient supply and the subsequent growth of entrapped organisms [17••]. The resultant lack of connectivity in heterogeneously dispersed microbial communities is readily demonstrable by significant metabolic activity increases with addition of water or crushing sediments in the absence of added substrates. Wetting a rock or smashing it to aid in sampling can induce such disturbance artifacts.

Subsurface systems selected for their geological conditions of hydrological isolation and maximum paleotemperatures of 120–145°C, conditions that could preclude microbial survival, were shown to contain no viable microbes by culture or biomarker analysis [18••]. The sedimentary microbial community at the time of deposition has been eliminated. Low levels of viable organisms such as sulfate-reducing Gram-positive *Desulfotomaculum*-like bacteria and methanogens were isolated from cores recovered from subsurfaces with hydrologic connectivity and lower paleotemperatures [18••]. These subsurface systems were recolonized by microorganisms within the past five million years after sterilization by the thermal event which occurred 40–45 million years ago. In another area careful geological analysis of cores from the Taylorsville Basin in Virginia indicated that the thermophilic bacteria isolated from this site most probably colonized the strata during the last major tectonic upheaval in the Jurassic [19••]. Iron-reducing thermophilic bacteria forming magnetic oxides isolated from the Taylorsville site have the temperature tolerance and metabolic activities compatible with the extant conditions [20••]. These conditions and these organisms are not found at the surface or at other places in the borehole. Similar anaerobic thermophilic organisms were recovered from the Piceance Basin in Colorado, which is isolated hydrologically, temporally and spatially from the Taylorsville Basin, but were entrapped under similar conditions. It is clearly possible to be very old and survive in the deep subsurface. This evidence indicates these microbes have survived *in situ* for at least several to 150 millions of years.

Metabolic processes in the subsurface

Redox reactions in the subsurface can be thought of as a competition for electron donors and acceptors that can conveniently be understood in terms of hydrogen concentrations [21]. The dominant anaerobic metabolic processes associated with the highest hydrogen levels are methanogenic. Reduction of sulfate, Fe(III), Mn(IV), and nitrate is associated with progressively lower hydrogen concentrations. Iron is one of the most abundant metals in the earth and increasingly iron is thought to play a key role in the subsurface microbial metabolism. A wide variety of organisms is now known to utilize iron reduction as a terminal electron acceptor for the oxidation of organic matter [22•]. Anaerobic oxidations can occur in the subsurface. Nitrate can be involved in an anaerobic iron cycle so the presence of oxygenated water is no longer required for iron oxidation [23•]. This may have important implications in the precipitation of radionuclides at anaerobic sites as oxidation can greatly increase solubility and mobility for nuclides like U, Tc for example.

One of the most exciting prospects in the research of subsurface microbial activities is the discovery of 'shuttles' whereby the electron accepting moieties localized in the subsurface pores with throats too narrow for bacteria to enter can transfer electrons between bacteria and the oxidized metals located in the pores. Lovely *et al.* [24••] demonstrated that humic substances, which are ubiquitous in the subsurface, can be used by some microbes as an electron acceptor for the anaerobic oxidation of organic compounds and hydrogen. These

humic substances also reduce less accessible electron acceptors such as insoluble Fe(III) oxides. The fact that 2,6-anthroquinone disulfonate (a model for quinones found in humates/humic acid) efficiently shuttles electrons between hydrogen, Fe(III) oxides, and can serve as a terminal electron acceptor suggests a mechanism for these humic substance stimulated activities. The humic 'shuttle' could be one of the most important features of anaerobic subsurface activities.

Stevens and McKinley [25] reported the surprising find of a basalt-based ecosystem in the deep subsurface that is independent of solar energy or other surficial energy inputs. This anaerobic subsurface lithoautotrophic system is able to sustain viable microbial ecosystems hundreds of meters below the surface and has implications for life anywhere there is liquid water and compatible temperatures. Evidence for the ecosystem included methane depleted in ^{13}C, the generation of considerably more hydrogen than could be accounted for by the organic carbon, the high ratio of autotrophic to heterotrophic bacteria, the generation of hydrogen from crushed basalt, and microbial growth in microcosms containing the basalt [25].

Granitic rocks may also have a deep subsurface microbiota. Microfossils found in calcite in a water-conducting fracture of 1800 million year old granitic rock from 207 m below sea level were analysed for ^{13}C and ^{18}O [26•]. The analysis showed the microfossils were enriched in carbon but low in ^{13}C suggesting strongly that the microbes were once metabolically active. The detection of ancient life in fissures suggests that modern life in these fractures could be intrinsic.

Even the sediments 150 m below the deep sea surface may harbor an active microbiota [27•]. The first evidence that microbial processes may influence alteration of the ocean crust has come from the correlation between glass alteration features (sites of the transformation between compressed sediment and basalt) and the presence of particulate nucleic acid [28•]. As yet, PCR of the isolated DNA extracts has not confirmed that the microbes are indigenous to the volcanic rock. The procedure that Giovannoni *et al.* [28•] developed for correlating mineralogy with microbial activity at the microscopic scale may represent a new technique applicable in the subsurface. We look forward to definitive analyses of deep-sea basalts with appropriate quality assurance to establish that the isolated microbes are indeed the extant microbiota.

Utilization of subsurface microbiota

The deep subsurface microbiota can be manipulated by judicious nutrient application to stimulate bioremediation. Addition of gaseous triethyl phosphate with methane and nitrous oxide to horizontal wells surrounding a trichloroethylene contamination site stimulated the fortuitous breakdown of trichloroethylene by subsurface methanotrophic microbes [29•]. These manipulations added 7% to the cost of the contamination clean up operation but resulted in degradation of 40% more of the contaminant as indicated by increased chloride, increased methanotrophic populations and decreased contaminants in the subsurface [29•]. Bioremediation may require augmentation with nonindigenous bacteria in addition to the nutrients.

Augmentation of the subsurface microbiota with nonindigenous bacteria could overcome some of the problems associated with hydrogeological and geochemical heterogeneities in limiting bioremediation effectiveness. To establish effectiveness of bacterial augmentation in the subsurface, tracer bacteria were selected with low adhesion properties so they could move freely in the subsurface and with 'labels' of nonclinical antibiotic resistance and by growth with nonradioactive ^{13}C glucose mass label [30•]. The detection of ^{13}C labeled bacteria after recovery from subsurface inoculation proved the most sensitive and discontinuities between the bromide non-reactive tracer, which moves freely, and the bacteria transport, which is effected by adhesion and pore throat size, were readily demonstrated. Initial results indicate that most of the bacteria are adsorded at the sites of injection but subpopulations can move rapidly. In addition to the physical and chemical heterogeneities of the aquifer, a biological heterogeneity must also be considered when modeling bacterial transport.

Implications for extraterrestrial life

The report by McKay *et al.* [31•] that the nanometer scale structures on the Martian meteorite recovered in Antarctica might indicate life stirred up an enormous excitement. Researchers focused their interest on the sampling of the Martian subsurface for homologues of the microbes that are found in the fractured layers of the Columbia river basalts.

From this brief review the iron-reducing bacteria, particularly those recently detected that are thermophiles, may have been of major importance in the ancient Earth and could thus have been important on our brother planet Mars. Thermophilic dissimilatory iron reduction with the metal as the terminal electron acceptor was demonstrated recently in anaerobic Gram-positive bacteria [32•] related to the *Bacillus-Clostridium* subphylum. This organism *Thermoterrabacterium ferrireducens* joins a group of bacteria that includes the anaerobic *Bacillus infernus*, which reduces Fe(III) with lactate and formate, and the aerobic archeon *Sulfolobus acidocaldarius*, which utilizes Fe(III) and elemental sulfur. *T. ferrireducens* is the first dissimilatory iron reducer directly isolated from a terrestrial geothermal area and it strongly suggests that anaerobic electron transport could be an ancient metabolic process.

Conclusions

With the quality assurance of tracers and microbial community and activity assessments it is now possible

to sample the extant microbiota of the deep subsurface. So what is going on down there? The microbes may be truly ancient and capable of remarkable feats of perseverance and starvation. Thermophilic dissimilatory iron reduction with 'shuttles' to bring electron donors to the microbes may be a very important life sustaining process. This microbiota can be manipulated and traced through geologic heterogeneities. Microbes can move and modify the rocks with their metabolism. The ocean floor sediments and crest may be a new frontier for exploring the subsurface biosphere, particularly if hydrogen can be generated abiotically from basalts at rates compatible with life so that they may have an energy source independent of the surface. Nothing we have learned from the terrestrial subsurface discourages us from searching for extraterrestrial microbial life.

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