

## **DUSEL: Window to the Subsurface Biosphere**

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The relatively recent discovery of deep subsurface microbial communities and what has been identified as a subsurface biosphere has opened a new scientific frontier where earth sciences, chemistry, physics and biology merge to provide insights into how life on this planet and even extraterrestrial life, may have originated and evolved over billions of years. The geological isolation of these deep subsurface microbial communities offers the potential to answer questions on the origin of life and its diversity as well as constraining the possibilities for life beneath the surface of Mars and other planetary bodies. In addition to the role of microorganisms in shaping the life forms on earth, their importance in the dissolution and formation of minerals is only now becoming recognized as geomicrobiology comes to the fore. Advances in our understanding of the origins, diversity, distribution, and function of microorganisms in deep, often extreme, subsurface environments will rapidly expand our knowledge of geomicrobiological and biogeochemical processes on Earth and beyond. The discovery of novel microorganisms from deep subsurface habitats provides opportunities for discovering new pharmaceuticals, processes for biochemical and chiral-specific synthesis, environmental remediation and energy production. Finally, a fundamental knowledge of subsurface biogeochemical processes and elemental cycling is critical for predicting the impacts of subsurface contamination and underground waste isolation and for development of subsurface remediation strategies, including the storage of radioactive waste and CO<sub>2</sub> sequestration.

The deep terrestrial subsurface is aphotic and the microbial inhabitants represent “dark life” functioning largely independent of sunlight and interactions with eukaryotes. They depend on energy and nutrients from kerogen (ancient organic matter), inorganic sources associated with the host rocks and associated fluids, and a range of other abiotic sources such as H<sub>2</sub> from radiolysis and hydrocarbon gases from crustal inorganic carbon sources. Microbial populations are typically characterized as sparse, isolated in tiny pores, slow growing/respiring (i.e., long doubling times), and sometimes starved or resource-limited relative to their surface-dwelling counterparts, and other times not. The deep earth, therefore, offers a wide variety of unique environments for molecular biology studies because of the opportunity to access populations that have been isolated from the surface environment for quantifiably long times (thousands to millions of years) and with different resource and population constraints. Hence they provide a particularly exciting opportunity for evolutionary studies.

Several key scientific questions have emerged from subsurface biogeochemical studies to date. These include the following questions, which are discussed in detail below.

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

A major obstacle to understanding the subsurface biosphere has been our limited ability to access the deep terrestrial environment, to acquire uncompromised samples, and to place our knowledge of isolated microorganisms into the context of the geochemical and hydrogeological processes. DUSEL would provide an unprecedented opportunity for geomicrobiological sampling and experimentation in the subsurface. The unique value of a dedicated subsurface science and engineering laboratory to geomicrobiological research is further explained below.

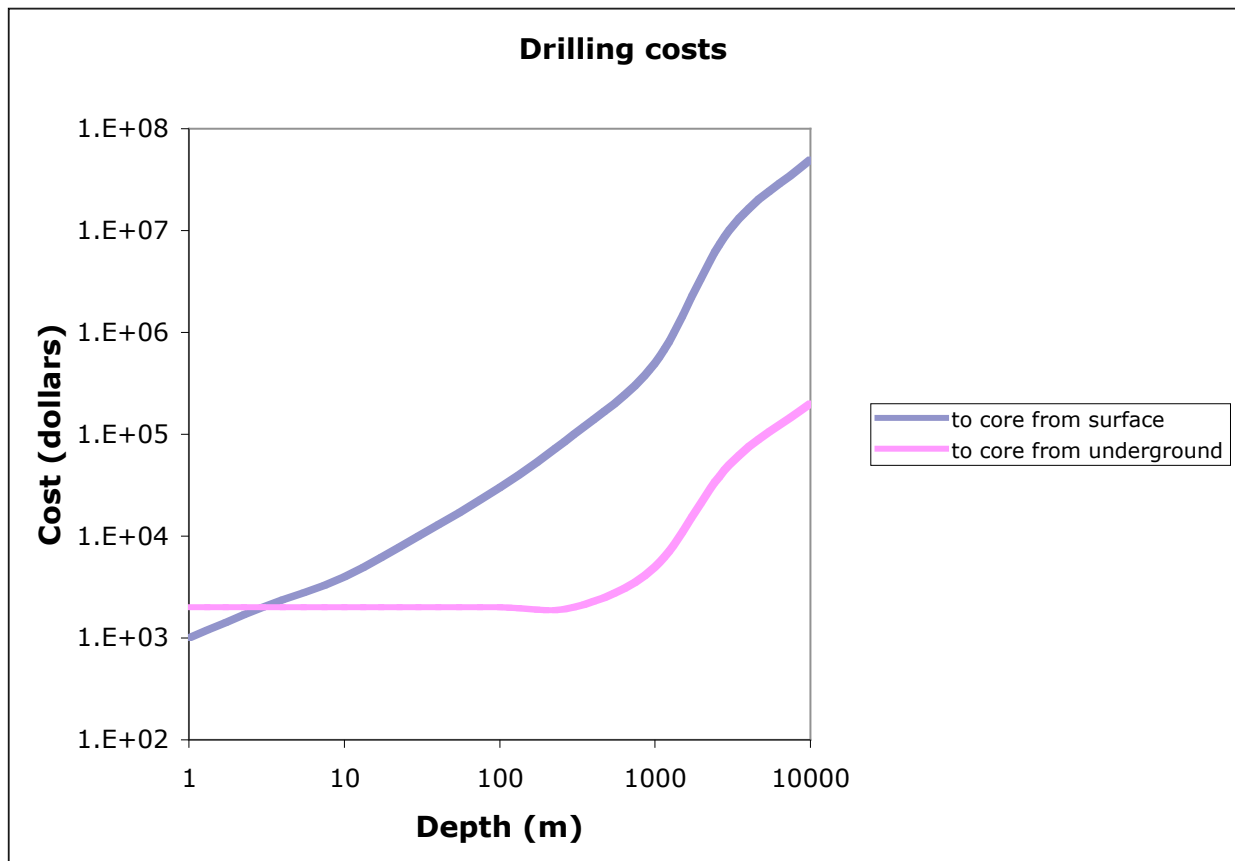
### **1. How deeply does life extend into the Earth?**

In a report from the American Academy of Microbiology (Nealson and Ghiorse, 2001), a major topic of discussion concerned “life in deep subsurface regions of the Earth’s crust where, until recently, life was not thought to exist”. The report goes on to state as the first major question: “What is the extent of the biosphere that exists deep below the Earth’s surface? When and how did it evolve? How much biomass lies there?” It is surprising perhaps that we know more about the surface of the Moon and Mars than we do about the deepest regions of our own biosphere (Nealson and Ghiorse, 2001). The existence of a deep subsurface biosphere has only gradually come to light over many decades; however, our scientific progress has been limited by sampling technology and availability of sampling sites. Until recently only a handful of samples from greater than ~1 km depth had been obtained and none had been collected without some degree of compromise due to the contamination inherent to drilling or mining. A theoretical sample from another planet must only traverse the sterility of space, whereas, a sample from the subsurface will have had multiple opportunities for contamination. Despite these difficulties, a picture of the deep subsurface biosphere is emerging. Estimates of its total biomass have been prepared (Onstott et al., 1998; Whitman et al., 1998), often producing totals that are comparable to the collective biomass of the much better known surface biosphere.

Given the heterogeneity of the deep subsurface in terms of thermal gradient, rock type, geothermal history, and hydrology, it is clear that no “one size fits all” definition of life and its limits in the deep biosphere will be possible. Rather, the lower boundary of the biosphere will vary by location depending upon the convergence of interrelated factors. Of the possible factors that could limit the distribution of life in the deep subsurface, the most uncompromising is temperature. The currently recognized highest temperature for the growth of a cultivated organism is 121°C (Kashefi and Lovley, 2003). Given the increase of the upper temperature limit for life in the literature from the 1950’s to today, one can surmise that the actual upper limit could be even higher, though probably by no more than a few degrees. Terrestrial geothermal gradients vary with location from ~8 to 60°C/km, suggesting temperature-induced depth limits for life of 2 to 12 km below land surface for mean surface temperatures from 0 to 25°C.

However, other constraints such as low availability of microbial energy sources to fuel cell maintenance and growth or high pressures (~10 MPa increase per km depth) may combine with high temperature to further delimit the biosphere. A fundamental requirement for life is liquid water; however, large portions of the subsurface may be devoid of liquid due to displacement by geologically or biologically produced gases. Thus, dry, gas-charged zones may represent barriers to the colonization of potential deep subsurface habitats following geological uplift, erosion, and cooling (Colwell et al. 2002).

Determining the lower limit of the biosphere and the factors that constrain it necessitates obtaining samples from great depth (5 km or more), which is a technically difficult and costly endeavor. The options are to drill from the surface or to drill from a deep underground platform such as the deepest level of DUSEL. Technical as well as cost considerations favor drilling from a deep underground site. Drilling to depths greater than a few hundred meters requires a rotary method that uses a drilling fluid to lubricate the bit and to remove the cuttings. The drilling fluid is a source of chemical and microbiological contamination and also must be pressurized to remove the cuttings. This pressure causes the drilling fluid to penetrate and thus to contaminate the pores and fractures within the core samples. If the drilling depth is reduced by placing the drill rig underground, e.g., at ~2.5 km depth in DUSEL, then the drilling fluid pressure and thus the potential for contamination are significantly reduced. The drill rig can be considerably smaller, as well. Drilling to ~5 km from the surface requires an enormous drill rig, whereas drilling and coring from a deep platform, as the mining industry does routinely, can be accomplished with a portable drill rig that can be deployed in a ~200 m<sup>3</sup> space. The quantity of drilling fluid and the microbial contaminants from drilling are both substantially reduced. These differences in the technical requirements translate into an enormous difference in costs (Fig. 1)



**Fig. 1.** *Comparison of costs for coring from the surface and from a stance 2.5 km underground.*

and significant gains in detection limits. Thus, probing the deep limits of life will be greatly facilitated by DUSEL, where the costs associated with making deep underground drill sites available will be justified by the combined needs of multiple scientific disciplines.

## **2. What fuels the deep biosphere?**

A growing body of evidence indicates that abiotic geochemical processes directly or indirectly provide fuel for deep subsurface microbial ecosystems that are independent from surface photosphere production. For example, H<sub>2</sub>, an important electron donor for chemolithotrophs, appears to be produced in the lithosphere by a variety of rock-water interactions (Stevens and McKinley, 1995; Chapelle et al., 2002; Lin et al., in press). The H<sub>2</sub> fuels chemolithoautotrophs, e.g. acetogens, which in turn excrete acetate that is utilized by heterotrophic anaerobes for sustenance in what is referred to as SLiME's, or subsurface lithotrophic microbial ecosystems (Stevens and McKinley, 1995). Organic substrates, such as simple hydrocarbons, e.g., methane, ethane, propane and butane, can also be generated by Fischer-Tropsch-like processes (Sherwood Lollar et al., 2002), and these can be readily utilized by known SO<sub>4</sub><sup>2-</sup>-reducing bacteria (Aeckersberg et al., 1991). Thermal oxidation of these simple hydrocarbons produces carboxylic acids that provide substrates for heterotrophic bacteria (Seewald, 2001).

Recently, water radiolysis fueled by natural radioactivity has been posited as a source of H<sub>2</sub> and also SO<sub>4</sub><sup>2-</sup> (Lin et al., 2005). The deep groundwater environments where these abiogenic H<sub>2</sub> and SO<sub>4</sub><sup>2-</sup> sources have been found also bear molecular and stable isotope evidence of indigenous SO<sub>4</sub><sup>2-</sup>-reducing bacteria (Baker et al., 2003; Boice et al., 2004). Thus, many subsurface environments appear to provide reductants and oxidants that are metabolized by indigenous microbes. In some cases, neither oxidant nor reductant has been exhausted, suggesting that some other factor limits cellular metabolism (Kieft et al., in press). Potential limiting factors include lack of essential inorganic nutrients or accumulation of toxic products of microbial metabolism and geological reactions, such as H<sub>2</sub>S and CO.

An important limitation common to all deep subsurface environments is space. Porosity is typically less than 1% and permeability is often less than 1 mDarcy (Onstott et al., 1998). Consequently, deep groundwater moves extraordinarily slowly and microbes on fracture surfaces depend on flow-limited nutrient flux. In the absence of appreciable flow, microbes may also create miniature zones depleted in substrates that can be replenished only by diffusion. Because biomineralization and biogas formation hinder the nutrient flux by closing pore throats, these microniches may go through cycles of growth, steady-state, and decay. The fact that so many of the bacteria and 16S rDNA clone libraries obtained from the deep subsurface appear to be spore formers hints at a strategy by which organisms in diffusion-limited habitats first grow, deplete their local nutrient supply, and then sporulate to survive. When local nutrient concentrations again become permissive, the spore then germinates and the cycle begins anew.

Any requirement for syntrophic interactions, i.e. in SLiME's, at very low microbial densities would further limit metabolism as energy flux resources would be shared. One of the primary

research goals of an underground microbiology facility is to understand the relationship between energy input and biomass for a volume of rock for which the total energy and mass balance can be closely monitored. Mining operations potentially disturb the local hydrologic environment, thus temporarily increasing nutrient flux, stimulating microbial growth and activity and enhancing microbial transport. Borehole sampling may also stimulate activity by the simple act of withdrawing fluid too suddenly. Borehole experiments designed to have the least impact on the local geochemistry, the energy/biomass balance and the flow rate combined with long period of observation are prerequisites for underground microbiology experimental facilities.

### **3. How does the interplay between biology and geology shape the subsurface?**

Microorganisms are affected by and interact with the environment within which they live. Likewise, an environment is affected by and interacts with the biota that inhabits it. These two phenomena are intricately interrelated and result in a specific cause and effect pattern between the biota and the environment. A change in one component will usually result in a response(s) in the other. Thus, one approach that is often sought in the field of microbial ecology is to study the microbe-environment interaction within an *in situ* context. That means attempting to understand both the microbial community (and/or microbial processes) and their geochemical and physical environment. The *in situ* aspect allows an examination of the dynamic relationship between biology, the geological setting, and the physical environment and how those dynamics affect the nature of microbe-environment interactions.

An obvious requirement for understanding and characterizing microbial ecology in the deep subsurface is appropriate access to properly examine the microbe-environment interaction within *in situ* conditions. Processes and interactions in the subsurface are slow, subject to many limitations and restrictions, and difficult to assess remotely. Yet, the subsurface is an extremely important microbial environment from a global perspective. Little is known about the role that microbes play in the diagenesis of earth's solid materials, in key rock-water interactions, or the possibility of subsurface ecosystems not reliant on photosynthesis (e.g., geogas). Over the past 15 years the ocean drilling program has made enormous strides in characterizing the microbial communities present in seafloor sediments and the ocean crust. Whether the deep subsurface microbial communities of the terrestrial subsurface are distinct or similar to those of the marine realm is completely unknown.

The DUSEL project would provide unprecedented access to the terrestrial deep subsurface, enabling examination of the resident microorganisms and their interactions with the environment. This could include characterization of the communities relative to the rock structure and tectonic history, quantification of biogeochemical processes and experimental manipulations to examine community or process responses. Some of the potential processes that could be investigated include mineral precipitation and dissolution reactions, geochemical evolution of groundwater (both long- and short-term), transport and migration of subsurface microbes, microbial adaptation and evolution in ancient sequestered groundwater, ecological genomics and gene transfer, responses to contamination (and to the presence of DUSEL), biofilm formation, function, and relation to fracture transmissivity, groundwater-rock reactions driving ecosystem energy flux (e.g., geogas), and other dynamic impactors such as physical factors (temperature and pressure), seismicity and time of hydrological isolation.

Because the DUSEL project enables long term (decades) access, the first comprehensive analyses of terrestrial subsurface life, its nutrient sources utilized by these microbes and its associated remnant biogeopolymers can be undertaken. This daunting, but important aspect of the project will be possible due to the extensive controlled sampling and monitoring that DUSEL facilitates and the long range multidisciplinary approach that has been the hallmark of this endeavor.

For the first time in the deep subsurface, a mass balance approach will be taken to account for all of the biomass (living and remnant, planktonic and sessile) that makes up life underground. Bulk elemental (including C, N and S) content and isotopic values will be assessed, and a continuous monitoring of  $^{14}\text{C}$  ages will be followed throughout the extraction procedure, including bulk and compound-specific  $^{14}\text{C}$  values. These measurements could be performed at a new, low level counting facility within DUSEL providing a unique opportunity of synergetic collaboration between geomicrobiologists and physicists. This approach will illuminate the age of the C in the active biomass, the available nutrient sources and the bioresistant macromolecular components that may be present. Chirality of the amino acids may prove a useful monitor of living and remnant protein, peptide, and free amino acids, and the general autofluorescence, aromaticity maturation, degradation profiles, and isotopic composition of both extractable and rock-associated organic matter will illuminate the progression of biomass degradation in the deep subsurface. It is difficult to predict, but important to document the range, extent, and molecular composition of the organic, nonliving mass in the deep subsurface—it is here that organic geochemistry meets microbial ecology.

#### **4. What can we learn from subsurface genes and genomes?**

What is the tempo and mode of evolution in subsurface organisms and how does microbial life evolve in (extreme) isolation? Organisms from the deep subsurface provide natural experiments for addressing central questions about the basis of evolutionary change. Genetic change in lineages and populations, the substance of evolution, results from three basic forces: mutation, natural selection, and genetic drift (the chance increase of some genes at the expense of others). Recombination of genotypes and migration of individuals play important accessory roles, by shuffling genes within individuals and populations and thus influencing the action of selection and drift. In the quiescent and patchy distribution of niches within the deep subsurface, all of these processes are expected to operate in ways that are distinct from the surface biosphere and perhaps in a manner that more resembles life on the early Earth.

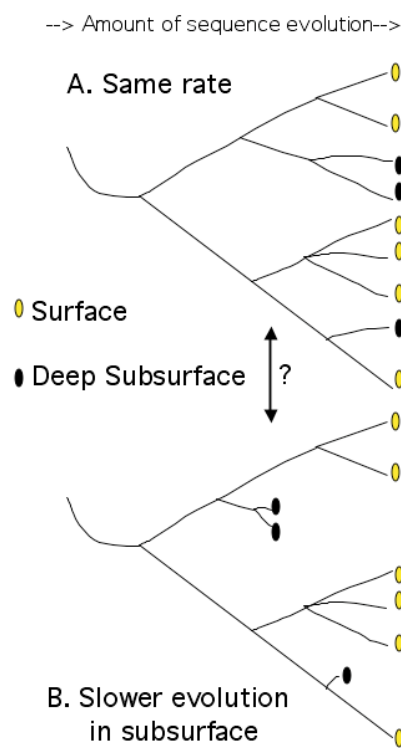
The ultimate source of genetic change is mutation, resulting from damage in DNA or errors in replicating DNA strands. Mutation is one of the most extensively studied of biological processes, not only because of its central role in evolution, but also because it is a critical element in disease and aging of humans, animals, plants. The rate of mutation and the spectrum of mutational types are affected by both external variables, mainly radiation and chemical factors, and intrinsic factors involving the replication system of a particular cell type. Based on the theoretical work often referred to as the “Neutral Theory of Molecular Evolution” (Kimura, 1983), we can estimate rates of mutation over long periods of time as the numbers of neutral changes occurring between two or more divergent lineages. “Neutral” changes are those not

affecting the performance of the organism; current knowledge of molecular function and the genetic code allows us to categorize sets of sequence changes as effectively neutral (for example, the changes within a protein coding gene that have no effect on amino acid sequence of the protein). To illustrate one long-standing idea that can be tested using gene sequences of subsurface organisms, slow rates of replication have been predicted to result in slower rates of neutral divergence on absolute time scales, due to lowered incidence of replication-associated mutation. The generation times in the deep subsurface are likely to be extremely long, due to long persistence of non-dividing cells that maintain low levels of metabolism under adverse conditions and/or ability of some lineages to form highly persistent spores. Additionally, a major source of mutation, UV irradiation, is absent, although other sources of radiation are likely to be elevated at least in some substrates. A clear cut prediction is that subsurface lineages will show depressed rates of evolution for neutral sites. These predictions can be refined considerably, for example by partitioning the mutational spectrum into changes that can be traced to DNA damage within spores between replication events (incorporated as mutations during the next round of replication) and those that accompany DNA replication. The most direct tests of altered mutational rates and patterns will depend on subsurface organisms with relatively close relationships to known surface organisms, such as the gram-positive *Arthrobacter* isolates or members of the *Desulfovibrio* group, among the most abundant cultivable microbes in deep subsurface communities (van Waasbergen et al., 2000, Sass and Cypionka, 2004) and low G+C gram positive species that dominate deep subsurface environmental clone libraries (Hinton, pers. comm. 2005). Statistical analyses that model sequence evolution along branches of a phylogenetic tree can, for example, test whether subsurface isolates of these groups are consistently slowed for mutations generally, or particular classes of mutations (Fig. 2). Harvesting DNA from subsurface organisms offers unparalleled opportunity to understand mutation in radically different environments and with exceptional life histories of long generation times.

Because subsurface microbes are expected to have highly isolated subpopulations founded by one or few colonizing cells, these lineages are predicted to show relatively frequent fixation of mutations with mildly deleterious effects on fitness, such as most mutations impacting the amino acid sequences of proteins functioning as enzymes. Thus some categories of changes (neutral ones) may be slowed whereas others are accelerated. Large differences among locations in degree of isolation should correspond to differences in these effects. Other predicted consequences of these high levels of genetic drift are loss of non-essential genes from the genomes. Local sub-populations are expected to be subject to strong natural selection with different pressures operating at different localities. Together both selection and genetic drift will thus tend to enforce genetic homogeneity within local colonization sites and divergence among sites, even ones separated by only very small distances.

Recent studies of community structure of environmental (surface) bacteria and archaea have documented surprising diversity of closely related genotypes occurring as complex assemblages in particular sites, with genotypic complexity compounded by unexpectedly high levels of genetic recombination (e.g., Whitaker et al. 2003, Tyson et al. 2004, Papke et al. 2004). Earlier views of geographically homogeneous clones with ubiquitous distribution and strictly clonal reproduction are thus being replaced by appreciation of the genotypic diversity and complexity among strains within a site and between sites, particularly in terrestrial habitats. A contrast is

provided by the extensive geographic distributions documented for widespread ecotypes of dominant marine bacteria (Rocap et al. 2002). In subsurface microbes, a general expectation is an extremely patchy population structure with rampant microdiversification and little homogenization. However, these expectations are tentative and the spatial component of population genetic structure will vary among sites according to permeability and structure of the subsurface matrix. Additionally, patterns are expected vary among subsurface microbes with different life histories and capacities for persistence under unfavorable conditions. In some groups, factors such as formation of long-lived spores, enabling long distance dispersal even through very slow or rare, could act as homogenizing forces. Even without spore formation, recent data indicate that bacterial and archaeal cells possess remarkable ability to survive very long periods of adverse conditions between opportunities for replication and cell growth (e.g., Price and Sowers 2004).



**Fig. 2:** Schematic of alternative phylogenetic results that would illustrate (A) similar evolutionary rates of deep subsurface and surface organisms or (B) slower divergences of deep subsurface organisms due to lower mutation or slow generation times.

The extremes of isolation and slow growth imposed by subsurface conditions are thus expected to be reflected in the dynamics of molecular evolution and population structure. By contrasting organisms with different life histories and substrates with different properties, the range of subsurface evolutionary processes can be elucidated. Achieving this research goal requires the expanded opportunity to sample microbial populations with precise information on relative

spatial position, substrate properties, and biogeochemical processes that only a DUSEL can provide.

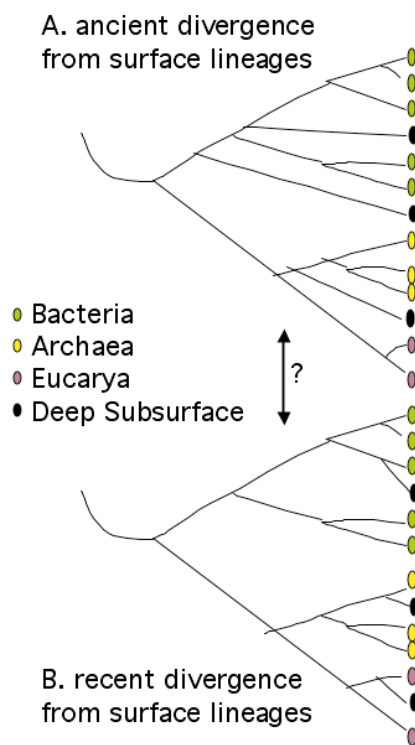
*What are genome "dynamics" under low population densities & slow growth?* Knowledge of microbes in the environment has been revolutionized in the last few years by massive amounts of genomic data and the analyses of these data. A general finding is that most bacteria and archaea undergo extensive gene acquisition and gene loss over time. Evolving lineages routinely acquire genetic and biological novelty that enable new ways of interacting with other organisms and the environment. Because of gene acquisition, closely related cell lineages can differ in presence of genes defining ecological breadth, suggesting that this process is a primary means of adapting to new niches. Although gene transfer is a frequent and overriding force in microbial evolution, sometimes bridging even the most divergent branches of the Tree of Life, our knowledge of its distribution and role in biological processes is still very limited. For example, specialized symbiotic bacteria sometimes show complete lack of gene acquisition over many millions of years, possibly because they have lost some genes for DNA recombination and/or because they have lost interactions with bacteriophage (e.g., Tamas et al. 2002). In contrast, some soil bacteria appear to show unusually high levels of recombination and DNA acquisition (e.g., Van Berkum et al 2003, *Streptomyces* genome), perhaps because they inhabit a densely populated and diverse habitat in which there is both opportunity and need for gene exchange, due to intensive "biowarfare" among different cell types. A clearer picture of where and why gene exchange and acquisition are most abundant would improve our broad understanding of how life evolves and how ecosystems function. It would also provide a foundation for more fruitful harvesting of useful genes and their products (e.g., antibiotics, chemotherapeutics, bioremediation agents) from naturally occurring organisms.

Where do deep subsurface organisms fit within the web of gene exchange that, we now know, encompasses the major lineages of bacteria and archaea, as well as bacteriophages? Subsurface organisms may belong to lineages that are largely sequestered from surface life and that harbor a high density of unique genes. As discussed in the preceding section, such physical isolation varies among locations, depending on the subsurface movement of fluids and particles (van Wassbergen et al. 2000). The low densities of subsurface organisms may preclude significant levels of gene transfer and may even be insufficient to maintain genetic machinery for acquiring and transferring genes. Thus, genomes of subsurface cells are expected to be relatively static, with little incidence of exchange. Bacteriophages, a primary source of genetic novelty in surface microbes (e.g., Pedulla et al. 2003), are usually disproportionately sparse where hosts are sparse, as expected since phage replication will be geometrically dependent on the rates at which particles encounter host cells. A lack of phage would push subsurface organisms further towards genomic stability. Current knowledge about the presence and persistence of phage particles in the deep subsurface is extremely limited, however, and recent studies on other systems suggest that relationship between phage densities and host densities can be affected by several factors such as changes in the life cycles of the phage (Sullivan et al. 2003). Finally, in the deep subsurface world, free DNA may avoid biodegradation for long periods; such free DNA could be a source of novel genes if taken up by cells.

Because the extremes of many subsurface environments impose unique selective pressures, the genomes of these organisms are expected to possess novel pathways and genes. Thus,

exploration of subsurface genomes will likely extend the range of known metabolism. Subsurface biota potentially contain some of the deepest branches of the Tree of Life, reflecting a role of the subsurface as a refuge during periods of global environmental adversity on the surface and/or the occurrence of niches resembling some of the Earth's earliest environments, particularly those involving fermentation of inorganic substrates. Genomic samples from the deep subsurface will provide extensive information on whether primitive life is indeed present and on what genes and features characterize such organisms if they are found (Fig. 3).

DUSEL potentially provides an opportunity for discovering of basal branches of the Tree of Life that will enhance the concurrent development of increasingly sophisticated tools for reconstructing the relationships among all parts of the Tree of Life. This topic now represents a very active area spanning computer science, biology and statistics; for example, the NSF now supports a large consortium dedicated to the development of computer algorithms and database structures for the elucidation of the complete Tree of Life. The complexity of bacterial and archaeal genomes, in light of extensive gene acquisition, is a central topic being addressed by numerous computational research groups. As a result we are poised to achieve a much more complete understanding of how genes move among organisms and environments as life extends itself across the Earth. The exploration of subsurface genomes, which are likely to fill missing links and to reveal sources of some gene families, will provide knowledge that is crucial to this landmark scientific accomplishment.



**Fig. 3:** Schematic of alternative phylogenetic findings that would illustrate (A) ancient divergence or (B) recent divergences of deep subsurface organisms from surface life. A mixture of both is also a possible finding.

## 5. Did life on the earth's surface come from underground?

The infant Earth – How was life sustained during the period intense meteorite bombardment? Life, as we now recognize it, is dependent on the presence of liquid water. Thus, estimates of when the Earth became habitable are closely tied to estimates of how rapidly the Earth cooled following accretion. When was liquid water available on Earth? More recent studies of oxygen isotopes and rare earth elements in a zircon recovered from Western Australia suggest that Earth's surface temperature may have cooled to the 100°C range by 4.4 billion years ago. If so, this would have provided an environment compatible with the variety of microbial life we now recognize soon after planetary accretion (estimated to be about 4.5 billion years ago). However, this time frame also corresponds with the period of intense meteorite bombardment during the Hadean Eon (4.6-3.8 billion years ago). The largest impact events would have boiled the oceans dry, raising the possibility that early surface life was repeatedly extinguished or that it survived in subsurface refugia.

One of the abiding mysteries of our existence is the origin of life. Since we have no deep understanding of this problem, we have no way to constrain the time span or circumstances necessary for life to emerge from prebiotic chemistry. However, if we consider the possibility that the emergence of life is a relatively rare event, then - if it was not repeatedly extinguished during the period heavy bombardment, life must have had a refuge from surface chaos. This is one of the more compelling reasons to consider the subsurface as the preferred, and possibly essential, habitat for early life on Earth. If subsurface environments of today resemble those of early Earth, it is also reasonable to entertain the possibility that the deep subsurface still harbors the most ancient of life forms.

What nourished early life? A question related to the availability of liquid water for life is the question of what energy fueled the early organisms of Earth. Today the most dependable energy source for life is the Sun. The emergence of photosynthesis profoundly altered the biosphere by capturing a virtually limitless supply of energy. Since the complex light harvesting machinery of photosynthetic organisms was invented long after the first life forms emerged, and freely available O<sub>2</sub> for respiration was a consequence of the even later emergence of oxygenic phototrophs, what nourished early life? This question can be divided into two interrelated questions – what were the sources of carbon and energy for primitive life? Considering the question of carbon, evidence for the abiogenic production by hydrothermal and photolytic processes is increasing and this combined with meteoritic and cometary importation of organic matter could supply the building blocks of life. As a fledging biosphere grew, however, it would eventually face a catastrophic shortfall of organic carbon and would have to evolve a means of converting inorganic carbon produced by volcanism into fixed carbon, thereby necessitating the invention of autotrophy.

The question – what source of energy sustained early life - is somewhat constrained by the requirements of constant supply and compatibility with a relatively unsophisticated energy harvesting machinery. H<sub>2</sub> is among the top candidates. This simple molecule is produced by a variety of abiotic geochemical and geothermal processes, and is the preferred substrate for a

variety of autotrophic anaerobic microorganisms. Many of these organisms are affiliated with the earliest radiations within the now-recognized universal tree of life (e.g., as inferred from comparative sequencing of the small subunit ribosomal RNA's). Microorganisms that might be representative of early H<sub>2</sub>-utilizing life forms include the methanogens (characteristically growing on hydrogen and CO<sub>2</sub>) and those organisms that couple the oxidation of hydrogen to the reduction of sulfate or oxidized iron (sulfate and metal reducers). Recent studies have shown these physiological groups to be abundant in the subsurface, and isotopic data now suggest that sulfate reducers were **present** on Earth as early as 3.5 billion years ago.

Another relevant type of anaerobic metabolism that exists, but is less well recognized is inorganic fermentation. This metabolism is similar to the familiar fermentation of sugars by yeast to yield CO<sub>2</sub> and ethanol. In the typical fermentation of an organic sugar, energy is recovered via rearranging the oxidation state on carbon. Relative to the parent compound (glucose), carbons in the products of fermentation are either more oxidized (CO<sub>2</sub>) or more reduced (ethanol). This type of reaction is also possible for inorganic compounds that are known to be abundant in subsurface, including elemental sulfur and sulfur dioxide. Both of these inorganic sulfur species can be used as the sole source of energy by many autotrophic anaerobes, releasing more oxidized (sulfate) and more reduced (sulfide) sulfur species as products of fermentation. A key advantage of this lifestyle is the requirement for only one chemical substrate as an energy source (the use of hydrogen as source of energy requires co-occurrence of a suitable electron acceptor, such as sulfate, CO<sub>2</sub>, or oxidized metals). Although the fermentation of partially oxidized sulfur species is the most well studied example of inorganic fermentation, it is possible that subsurface life may more generally take advantage of this energy source.

## **6. Is there life as we don't know it?**

Knowledge of life on Earth has expanded as previously unknown parts of the biosphere have been explored and as new technologies have broadened the search for life. As examples, the discovery of the deep sea hydrothermal vents led to the descriptions of new phyla of animals as well as novel microorganisms, and the application of molecular, sequence-based tools for characterizing microorganisms has resulted in a spectacular expansion of the known biodiversity of microorganisms. These molecular approaches have produced the widely accepted Tree of Life, based on small subunit ribosomal RNA gene sequences, with the majority of the major branches (phyla) of this tree comprising microbes that have not been cultivated in the laboratory. Thus the majority of life on Earth was unknown to science less than 20 years ago. This expansion of known biodiversity will likely continue with exploration of the deepest regions of the Earth's biosphere and continued innovation in biological techniques.

One can also imagine forms of life that would not fit easily into our present concepts of life, e.g., ones that do not conform to the DNA-RNA-protein dogma, or that function with alternate forms of these macromolecules. For purposes of this discussion, these alternate life forms are termed "life as we don't know it." These imaginative exercises are usually performed in the context of astrobiology, i.e., study of the potential for life on other planets and planetary bodies, especially those of other stars. However, life as we don't know it can also be considered for terrestrial

environments. If life as we don't know it exists on Earth, then the most inaccessible regions of the biosphere, e.g., the deep subsurface, may be the most likely habitats. The likelihood of this may be remote, but nonetheless, the deep terrestrial subsurface can be used as an analog for extraterrestrial environments and as a proving ground for instrumentation that tests for life as we don't know it in remote, inaccessible environments.

Many alternative chemical structures of biological molecules have been imagined and even synthesized and tested. For example, nucleotides and amino acids with opposite chirality to known forms require only a minor leap of imagination. Amino acids beyond the textbook 20 required amino acids can also be imagined. Indeed, our list of amino acids encoded by known nucleic acids has already expanded to include selenocysteine and others. Alternate nucleic acids have also been developed and tested, e.g., novel purines and pyrimidines, that form new base-pairings and yet are replicated by conventional polymerases (Benner and Sismour, 2005). Alternative electron transport systems that generate an electron transport system across a membrane have also been devised.

One can search for life by seeking the textbook hallmarks of life, i.e., metabolism, structure, etc. Metabolism depends upon and also forms chemical disequilibria, i.e., fluxes and gradients of redox-sensitive chemical species. Search for such disequilibria is the primary strategy of biogeochemists, whether they're investigating the terrestrial subsurface or sending probes to Mars. Biogeochemists look for gradients and fluxes of reductants ( $H_2$ ,  $H_2S$ , organic C compounds, etc.) and oxidants ( $O_2$ , nitrate, sulfate,  $Fe_3^+$ , etc.) and products of biologically mediated redox reactions (methane, etc.). Indeed, this approach has led to the finding of new forms of metabolism on Earth, e.g., dissimilatory iron reduction in the 1980s and anaerobic ammonia oxidation (ANAMOX) in the 1990s. This energetic metabolism-based search for life may be the broadest of all and may lead to the discovery of more novel forms of metabolism in the subsurface, as well as in extraterrestrial environments.

### **The need for DUSEL as a dedicated, deep underground facility for biology.**

Progress in addressing these questions has been severely hampered by the absence of a dedicated program and a dedicated facility for spatially extensive, long-term subsurface sampling and experimentation. With the exception of the ocean drilling program where such a dedicated program has been recently established, all subsurface scientific investigations have been compromised by sampling constraints. Nearly all microbiological studies of the terrestrial subsurface that have relied on drilling/coring studies for sampling have been piggy-backed onto projects with primary purposes other than science, e.g., petroleum and natural gas exploration. Others that have relied upon active mining excavations are entirely dependent upon the sometimes capricious generosity of the mine management. While some scientific questions may call for sampling in economically important geological formations, the majority do not. Basic research into the nature and extent of subterranean ecosystems requires access to pristine, undisturbed environments that are selected based on physical and chemical conditions that are expected to support indigenous microbes, including conditions at the extreme limits for life support. The few boreholes that have been drilled exclusively for geomicrobiology have been relatively shallow (<500 m) due to cost constraints. Moreover, the borehole approach yields relatively limited data in that sampling is inherently one-dimensional. Sampling in existing

mines and waste repositories allows spatially and temporally expanded sampling; however, these studies have been hampered by mine and repository activities. Underground laboratories exist outside the U.S., but these are relatively shallow and/or devoted to other purposes, e.g., waste repository performance (Aspo Hard Rock Laboratory, Sweden, depth, ~0.5 km) or physics (Gran Sasso, Italy; Kamiokande, Japan; SNO Lab, Canada; and others). A dedicated, deep underground laboratory in the U.S. will enable 4-dimensional access (X, Y, and Z directions plus time) with scientific investigation, including biology, as the primary objective. Microbes in the deepest regions of the biosphere exist in low numbers and have heterogeneous, patchy distributions that require spatially extensive sampling schemes that cannot be achieved by drilling from the surface. Continuous access will allow repeated sampling from a single site, e.g., to monitor conditions in a flowing borehole as they equilibrate with those of the water in the rock matrix, fractures, or aquifer being sampled. Unfettered spatial and temporal access will enable studies of coupled flow and transport experiments under the unique conditions of the deep subsurface.

Cost also weighs heavily in favor of a dedicated, deep underground facility for biological study. Studies of the terrestrial deep subsurface biosphere have been funded primarily by the NSF, NASA, and DOE. However, since the demise of the DOE's Subsurface Science Program approximately 10 years ago, there have been no programs devoted to basic biological investigation of the subsurface. Funding by NSF and NASA has enabled increasingly deep studies, but these grants have all been for sampling in existing mines. Funding has supported individual scientists and students but has not invested in infrastructure. Compelling scientific questions regarding the nature of the biosphere can be addressed only by deep sampling and deep in situ experimentation. It is fortunate for the field of geobiology that there are other disciplines with major scientific questions that can be addressed only by going to great depth. The NSF can therefore obtain maximum scientific benefit from funding the construction of a deep, dedicated underground scientific facility by considering the needs of biologists along with those of physicists, geologists, and engineers. Scientific considerations dictate that biologists go deep and cost considerations dictate that investment in deep underground infrastructure such as a DUSEL serve the long term needs of multiple scientific disciplines.

## **Summary**

Probing the deepest extent of the biosphere will expand our fundamental understanding of the limits of life and of its biodiversity. Terrestrial deep subsurface habitats are fundamentally different from other thermal environments, e.g., deep sea hydrothermal vents; and the diversity of microbes and the biogeochemical processes that they mediate represent unique adaptations to extreme conditions. The extent to which life can persist under the extreme conditions at depth on our own planet can also inform our understanding of the potential for life on other planetary bodies. Practical benefits may also ensue from exploration of the deep biosphere. Extremophiles from other environments have yielded biotechnological treasure; likewise, the secrets held in the deep biosphere may enrich our lives.

The American Academy of Microbiology identified the establishment of more field laboratories for geobiological research as a major recommendation (Nealson and Ghiorse, 2001). DUSEL would fulfill this need for subsurface geobiology by providing a unique opportunity to overcome

accessibility and intellectual obstacles by offering the ability to conduct long-term experiments with ready access to on site instrumentation for real-time detailed biological and chemical interrogations. As such, DUSEL will be a uniquely valuable facility for subsurface geomicrobiology research.

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