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The deep, hot biosphere: Twenty-five years of retrospection

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Twenty-five years ago this month, Thomas Gold published a seminal manuscript suggesting the presence of a “deep, hot biosphere” in the Earth’s crust. Since this publication, a considerable amount of attention has been given to the study of deep biospheres, their role in geochemical cycles, and their potential to inform on the origin of life and its potential outside of Earth. Overwhelming evidence now supports the presence of a deep biosphere ubiquitously distributed on Earth in both terrestrial and marine settings. Furthermore, it has become apparent that much of this life is dependent on lithogenically sourced high-energy compounds to sustain productivity. A vast diversity of uncultivated microorganisms has been detected in subsurface environments, and we show that H₂, CH₄, and CO feature prominently in many of their predicted metabolisms. Despite 25 years of intense study, key questions remain on life in the deep subsurface, including whether it is endemic and the extent of its involvement in the anaerobic formation and degradation of hydrocarbons. Emergent data from cultivation and next-generation sequencing approaches continue to provide promising new hints to answer these questions. As Gold suggested, and as has become increasingly evident, to better understand the subsurface is critical to further understanding the Earth, life, the evolution of life, and the potential for life elsewhere. To this end, we suggest the need to develop a robust network of interdisciplinary scientists and accessible field sites for long-term monitoring of the Earth’s subsurface in the form of a deep subsurface microbiome initiative.

geobiology | biogeochemistry | hydrogen | subsurface | thermophiles

A quarter-century ago this month, Thomas Gold, an Austrian-born astrophysicist from Cornell University, published a paper in these pages entitled simply, “The deep, hot biosphere” (1). In this paper, followed by a book of the same title (2), Gold suggested that microbial life is likely widespread throughout Earth’s subsurface, residing in the pore spaces between grains in rocks. Furthermore, he speculated that this life likely exists to a depth of multiple kilometers, until elevated temperature becomes the constraining factor. Gold hypothesized that life in subsurface locales is supported by chemical sources of energy, rather than photosynthetic sources, upon which surface life ultimately depends (1). The nutrients that support this subsurface life are provided by both the migration of fluids from the depths of the Earth’s crust and the host rock itself, which contains both oxidized and reduced minerals. Although it is likely to be all microbial, Gold posited that the mass of subsurface life in this little-known biosphere was comparable to that present in surface environments. Gold thought that if there is life at depth, the rocks that have

(or could produce) “hydrogen (H₂), methane (CH₄), and other fluids... would seem to be the most favorable locations for the first generation of self-replicating systems,” keenly aware that “such life may be widely disseminated in the universe” (1). Moreover, Gold hypothesized that hydrocarbons and their derived products fuel chemosynthetic subsurface life and that these hydrocarbons are not biology reworked by geology, but, rather, geology reworked by biology (2).

Although he did not have a doctorate, Gold (1920–2004) was highly recognized as a scientist, as evidenced by receiving a Gold Medal of the Royal Astronomical Society (1985) and a Humboldt Prize (1979); membership in the National Academy of Sciences (1974); and induction into the American Academy of Arts and Sciences (1974), the Royal Society (1964), the American Geophysical Union (1962), and the Royal Astronomical Society (1948), among others. As an author of ~300 scholarly papers, Gold had a penchant for contributing his thoughts to fields well beyond his own of astrophysics. In the foreword to Gold’s book *The Deep Hot*

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Biosphere, the theoretical physicist Freeman Dyson wrote, “Gold’s theories are always original, always important, usually controversial—and usually right” (2). Stephen Jay Gould considered Gold as “one of America’s most iconoclastic scientists” (3). Gold’s colleague and frequent coauthor Hermann Bondi, whom Gold met as a fellow “enemy alien” in an internment camp at the start of the Second World War, penned that, “Thomas Gold was a most unusual scientist. Brilliant in his ideas, unconstrained in the range of his interests, rock solid in his use of the fundamental laws of physics to make the most surprising deductions from them, making himself at home in fields of science he had no connections with before...” (4). Building on this sentiment, astronomer Steve Maran commented, “Unlike most scientists who are content to pursue the advancement of knowledge in small, incremental steps, Gold came up with new ideas by starting from the original principles” (3). Gold himself said, “In choosing a hypothesis there is no virtue in being timid... (but) I clearly would have been burned at the stake in another age” (3). Gold also took issue with the limitations of peer review, where established scholars pass judgment on new ideas and new papers before publication, which in turn rewards incremental steps, but few bold ideas that provide breakthroughs. Evidence of the effects of peer review, in Gold’s view, comes from the sheer volume of great ideas that came out of the first half of the 20th century relative to the latter half (3).

Gold believed that biology is just a branch of thermodynamics, being supported by energy in disequilibria in chemical reactions that would otherwise equilibrate if they were not held up by kinetic or mechanistic traps (5). To this end, the chemical energy available inside a planetary body would have been the first energy source, and solar energy, carbon fixed through photosynthesis, was a later adaptation of life (3). Although revolutionary in the infancy of the proposal, and not entirely unfounded in the scientific literature (6), informatics, geochemical, and isotopic evidence now provide evidence to support this view (7). Controversially, Gold also believed that oil and gas, which have fueled modern industrial nations for the past century, were born out of the Big Bang and were incorporated into the Earth’s crust at the time of the Earth’s coalescence 4.5 billion years ago. Gold arrived at this conclusion from the humble observation that meteorites and other planetary bodies contain abundant and compositionally diverse hydrocarbons (e.g., the methane lakes of the Jovian moon Titan), begging the question as to why hydrocarbons in the deep subsurface of Earth need to have a different origin (2). Bondi thought that this proposal in geology was Gold’s most important intervention to a field outside his own (4). Although speculation surrounding the formation of oil and gas has resulted in significant debate within the geosciences, the idea of abundant abiogenic hydrocarbons percolating upward had perhaps an even more profound influence on the biosciences by providing impetus to understand how such compounds could fuel a deep, hot biosphere since early in Earth history. Much of the debate concerning the presence of mantle-sourced hydrocarbons has been settled since Gold’s initial proposals, including evidence that mantle-sourced hydrocarbons in crustal environments are likely to be far less abundant than originally proposed (8, 9).

Regardless of present-day evidence indicating that light hydrocarbons in the crust are unlikely to have originated in the mantle, Gold pioneered the notion that such hydrocarbons (regardless of source) could sustain life in the subsurface to known depths of 10 km and possibly down to 300 km, if the temperature was below a hypothetical life-maximum of 150 °C (10). At higher temperatures, deeper within the crust/mantle, “cracking” of hydrocarbons could

release volatiles such as hydrogen (H₂) and methane (CH₄), which could percolate upward to thermal regimes that allow for life, thereby providing fuel. If true, H₂ or CH₄ sourced from abiogenic hydrocarbons, and the life that is dependent on these compounds, could be widespread in the subsurface of not just Earth, but also other planetary bodies (11–13). Indeed, H₂ has become a major focal point for microbiologists studying the deep subsurface (14, 15) and has been suggested to be the fuel that supported the earliest forms of life on Earth (16–18).

Gold’s deep, hot biosphere contribution challenged paradigms in subsurface science, petroleum research, the origin and evolution of life, and the search for life on other planets. Some argue that aspects of his ideas are highly flawed (e.g., the source of hydrocarbons in subsurface environments), whereas others argue that his ideas and the concept of a deep, hot biosphere make perfect sense. Regardless of one’s personal opinion, Gold’s ideas on the deep, hot biosphere have had a tremendous impact on scientific discourse, with the article being cited >325 times (Web of Science), and the book remains a top seller on [Amazon.com](https://www.amazon.com). Fig. 1 shows how the work continues to be highly cited by numerous fields outside of his field of astrophysics, indicative of its broad reach. Here, we provide an overview of new insights into the deep, hot biosphere developed over the past 25 years. These insights were made possible by the development of new tools and technologies and increased and better access, as well as enlarged interest—all of which can be traced (at least in part) to Gold’s pioneering paper. Particular emphasis is placed on the nature of microbial communities in subsurface water-rock-hosted ecosystems and their extent and diversity, and new insights into processes that sustain this life.

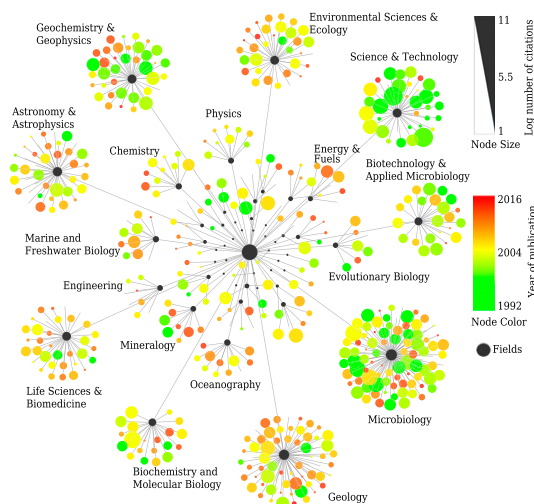


Fig. 1. Citation network of Gold’s 1992 “The deep, hot biosphere” publication (1). The citation index for Gold (1992) was downloaded from the ISI Web of Knowledge database (apps.webofknowledge.com/) and consists of 327 citations as of November 15, 2016. Citations were grouped by subject fields, or Web of Science Categories (WC). If multiple fields were given for a citation, all were considered in the network. Publications are shown as colored nodes, where Gold’s publication is the large center-most black node, and the others are colored according to year of publication (scale given on right) and sized according to the number of citations each has received (log scale given on right). Publications are grouped by subject field (black nodes), and those with more than five publications are labeled. A force-directed layout in Cytoscape (Version 3.2.0) was used to generate and view the citation network.

Energy at the Interface Between the Biosphere and the Geosphere

Gold controversially suggested that hydrocarbons originating in the subsurface (particularly from the mantle) or substrates derived from these hydrocarbons provide a source of energy capable of supporting a deep, hot biosphere. Although the merits of this hypothesis have been heavily scrutinized and largely proven false (discussed below), it is now abundantly clear that microbial life inhabits the subsurface and appears to be generally dependent on lithogenic substrates produced from water–rock interactions. H_2 and CH_4 derived from mantle sources are now known to contribute minimally to their overall abundance in deep terrestrial subsurface settings (8, 9), but, importantly, Gold's ambitious treatise set forth a generation of researchers that have now documented the nature of subsurface biospheres and the geologically sourced energy substrates that sustain them. Gold rightly drew comparisons from then-recent observations at hydrothermal vents that chemical disequilibria involving H_2 and CH_4 supported a diversity of chemosynthetic microorganisms that may also exist at depth in fracture zones. H_2 , in particular, has drawn considerable attention in the following 25 years as a major reductant for chemosynthetic life and is now known to be predominantly abiologically sourced via (i) radiolytic splitting of aqueous fracture fluids in granitic rocks (19–23); (ii) reduction of water by iron-bearing minerals in basalts and peridotites (14, 15); and (iii) radical-based splitting of water driven by the physical shearing of silicate minerals through a “mechano-radical” mechanism (24, 25) (Fig. 2). It is also possible that some H_2 supporting subsurface lithoautotrophic primary production derives from H_2 produced at depth by processes akin to those suggested by Gold 25 years ago. Regardless of the mechanism of formation, this lithogenic H_2 is abundant in Precambrian terrestrial formations (23, 26) and fuels ecosystem level primary productivity both in subsurface and surface environments impacted by subsurface processes such as geothermal and hydrothermal systems (27–30).

The ability to metabolize H_2 is pervasive in the microbial world, with ~30% of taxa with available genomes encoding for [FeFe]-, [NiFe]-hydrogenase, or [Fe]-hydrogenases (31), the principal enzymes involved in H_2 metabolism (31). An analysis of [FeFe]- and [NiFe]-hydrogenase homolog abundances in community genomes from subsurface environments reveals nearly an order of magnitude more copies per mega-base pair of sequence relative to those from surface environments (Fig. 3), consistent with the

central role of H_2 in the energy metabolism of subsurface life. Importantly, high concentrations of H_2 can also drive the abiotic hydrogenation of CO_2 through a process sometimes referred to as Fischer Tropsch synthesis (32). Indeed, at high temperatures, hydration of olivine and the H_2 produced has been shown to catalyze the abiotic reduction of aqueous CO_2 to formate ($HCOO^-$), which can then equilibrate to carbon monoxide (CO) (33). Intriguingly, genomes of subsurface microbial communities are also enriched in genes allowing for the use of $HCOO^-$ and CO under anoxic conditions, formate dehydrogenase and nickel-dependent CO dehydrogenase, respectively, compared with genomes from surface communities (Fig. 3). In contrast, molybdenum-dependent CO dehydrogenase was not found to be enriched in subsurface communities relative to surface communities, which may be due to limited Mo availability in anoxic and possibly sulfidic conditions present in many subsurface locales (34).

Methanogens, organisms with metabolisms suggested to be the most “primitive” among those available in extant life (18, 35), combine H_2 with CO_2 to drive energy metabolism and produce CH_4 as a metabolite. Methanogens use an ancient pathway of CO_2 fixation termed the reductive acetyl CoA pathway (35), which involves a number of Ni- and Fe-dependent enzymes. Chief among these is the acetyl CoA-synthase (ACS), which is also enriched in subsurface microbial communities relative to those found in surface environments (Fig. 3). The acetyl CoA pathway proceeds along a reaction pathway that involves a number of intermediates, namely, CO and $HCOO^-$, that also can be produced during the abiotic hydrogenation of CO_2 in the production of CH_4 and other hydrocarbons (33). Minerals that catalyze the abiotic reduction of CO_2 often contain copious amounts of Fe and Ni, similarities that have led to the hypothesis that the emergence of biological catalysts (e.g., CO-dehydrogenase, ACS, and [NiFe]- and [FeFe]-hydrogenase) involved in CO_2 reduction via H_2 took place in a subsurface environment where these minerals were abundantly available (35, 36). The most primitive methanogen by phylogenetic reconstruction is *Methanopyrus kandleri* (37), a hyperthermophilic organism isolated from a deep sea hydrothermal vent that grows at temperatures up to 122 °C (38). The physiological characteristics of this organism, its ecology, and its primitive nature are consistent with the notion of a deep, hot biosphere.

The Diversity of Life in the Deep Subsurface

Initially, we learned about the deep, hot biosphere from cultivation of taxa in fluids sampled from oil wells, mines, caves, and geothermal and hydrothermal environments, among others (39–42). Since Gold's call to probe the deep biosphere, increased access and sampling of subsurface environments in terrestrial and marine environments through drilling projects and increased collaboration with industrial partners have resulted in substantial expansion of the known microbial taxa in these environments. Furthermore, application of molecular approaches has resulted in the discovery of a plethora of previously unknown microbial lineages, particularly within the archaeal domain (43–49). Although a precise definition of what depth below the surface is considered “deep” is system-dependent, constraining the limits of the deep, hot biosphere to those environments that exhibit temperatures higher than ~50 °C and are >1 m below the surface [as is used elsewhere to operationally define the “deep subsurface” (50)] provide a metric from which to compare like environments.

Although there are a number of similarities between marine and terrestrial subsurface environments, important differences

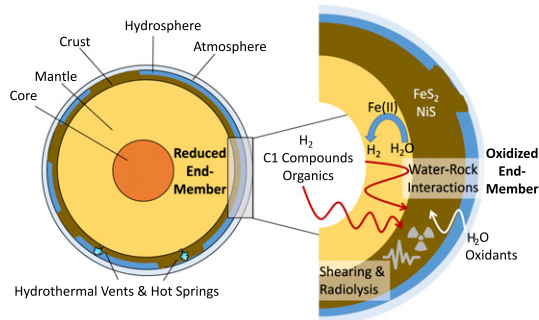


Fig. 2. Generalized schematic of the subsurface crustal environment and interacting forces that produce habitable conditions in the subsurface. Inset shows a magnified view of the interactions occurring at the surface–subsurface interface, and mechanisms of H_2 production in the subsurface that are discussed in the text are highlighted.

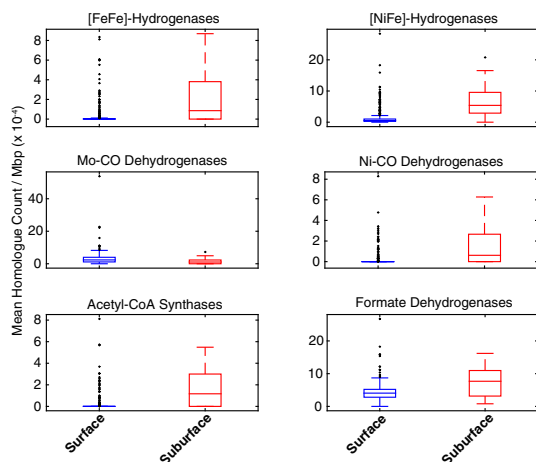


Fig. 3. Average abundances of key metabolic proteins in surface and subsurface metagenomes. Average abundances are given as the number of positive BLASTp searches found for each environmental metagenome in NCBI's metagenome ftp server (<ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/metagenomes/>). Search bait sequences for each functional gene were used after empirically validating the appropriate E-value cutoff that would only include homologous protein sequences in search results. Average abundances were normalized by the total sequence length (in base pairs) of each metagenome. Metagenomes without any hits to the six functional genes were not included in the results. Companion metadata were used to group metagenomes into surface and subsurface categories. Only environmental (e.g., nonengineered and non-host-associated) metagenomes were used in the analyses. Metagenomes were classified as surface or subsurface based on available metadata in which subsurface environments were defined as groundwater, oil reservoir, rock porewater, or marine sediments > 1 m below sea floor, and surface environments included all others.

also exist. Most conspicuously, the abundance of dissolved organic carbon (DOC) that is available to support heterotrophic populations differs between the two. The burial of photosynthetically derived organic carbon in marine subsurface environments supports heterotrophic populations at significant depths (51, 52), whereas studies of the terrestrial subsurface have largely focused on systems that are generally removed from surface influences. Although much of the deep marine sediments recovered from drilling programs are below the thermal temperatures that we define as hot (50 °C), recent estimates have suggested that ~34% of total ocean sediment volume exists in the temperature range occupied by thermophilic microorganisms (40–100 °C), with an additional 5% at temperatures theoretically capable of supporting life [100–120 °C (53)]. Clearly, the definition of deep is contextually dependent and has consequences for the physicochemical characteristics of such systems. Regardless, the defining features of environments hosting a deep, hot biosphere are that they are removed from surface photosynthetic processes by at least one or more steps (following ref. 50), have elevated temperature, and are generally anoxic. Although a number of other definitions can be used, this framework is used to guide the following discussion of the deep, hot biosphere.

In terrestrial settings, studies within mines (43, 45, 54, 55) at depths of between 10^2 and 10^3 m below the surface and borehole drilling into hydrothermal environments (15) have provided critical information on the nature of the deep, hot biosphere. Likewise, ocean-drilling programs in areas associated with hydrothermal activity have provided equally important insight into the nature of life in deep, hot marine environments (44, 48, 56–59). These

studies reveal a unique and considerable diversity of both cultivated and uncultivated lineages of Bacteria and Archaea that predominate deep thermal subsurface environments. Archaea that were detected in subsurface mine samples in South Africa and Japan include representatives from candidate divisions that have now been given the epithets of “Bathymarchaeota,” “Hadesarchaea,” and “Aigarchaeota” (59–61). Deep borehole fluids from Idaho and South Africa contained significant populations of thermophilic, methanogenic Archaea (15, 62). Considerable bacterial diversity has also been detected in thermal subsurface environments and includes members of the Aquificae, Firmicutes, δ -Proteobacteria, and Nitrospirae, among other proteobacterial groups and numerous uncultivated candidate divisions (46, 49, 62–66). Similar microbial taxa have been observed in deep, hot marine subsurface systems in addition to Archaea from the Archaeoglobales, Thermococcales, and anaerobic methanotrophic archaea (ANME) lineages and bacteria related to Thermotogae, Chloroflexi and the “Aminicenantes” (formerly OP8), among others (44, 48, 56–58). Although the previous 25 years of work have revealed abundant, novel taxonomic diversity in deep, hot subsurface environments, accompanying physiological diversity was less forthcoming until recent developments in high-throughput, -omics-based approaches.

Omic Insights into the Physiological Capacity of the Deep Subsurface Microbial Biosphere

The oligotrophic nature of subsurface environments selects for microorganisms that thrive on minimal energy gradients provided by available redox couples in highly reducing, low-oxidant environments. A subset of these environments that are removed from surface carbon input have been collectively termed subsurface lithoautotrophic microbial ecosystems (14). A lack of available surface-derived DOC likely necessitates a general capacity for autotrophy that is fueled by lithogenic energy sources, such as abiogenic H_2 . The prevalence of methanogenic Archaea in many of these systems is not surprising, because the H_2/CO_2 redox couple for biomass production typically dominates environments with minimal energetic gradients (67).

The prevalence of autotrophic sulfate-reducing archaea and bacteria (SRA/B) is consistent with the thermodynamic favorability of the H_2/SO_4^{2-} redox couple in the deep subsurface and the availability of these compounds for use. Key differences in the origin of SO_4^{2-} in marine and terrestrial subsurface settings exist, but its prevalence nevertheless leads to commonalities in the physiologies that support microorganisms in these environments. For instance, in the terrestrial subsurface, SO_4^{2-} is likely produced via oxidation of pyrite by radicals produced during radiolytic splitting of water—a process that was recently shown to play a key role in supporting sulfur-based microbiomes in the South African subsurface (68). In contrast, marine sediments and the underlying bedrock feature an abundance of sea-water-derived SO_4^{2-} (originally from weathering of continental sulfides). Indeed, SO_4^{2-} is a predominant electron acceptor in anoxic marine subsurface sediments (52). Like methanogens, autotrophic SRA/Bs fix CO_2 using the acetyl CoA pathway and also tend to use formate and CO as carbon and/or electron donors (69). The prevalence of key proteins involved in H_2 , CO, formate and the acetyl CoA pathway of CO_2 fixation encoded in the genomes of subsurface communities (Fig. 3) relative to surface communities is consistent with these observations.

Gold suggested that if the “archaeobacteria” (now Archaea) were indeed the most primitive of organisms and were largely

thermophilic, that they may have “evolved at some depth in the rocks . . . spread laterally at depth, and they may have evolved and progressed upwards to survive at lower temperatures nearer the surface” (1). It is intriguing to note then that the current revolution in archaeal genomics is, in part, driven by the discovery of numerous archaeal “subsurface” lineages, many of which provide key insight into the evolution of all Archaea, if not all life. Metabolic reconstructions from genomic representatives of several Archaea, initially detected in subsurface gold mines, have provided support for the importance of autotrophy and H_2 /C1 (one-carbon compounds) metabolism. *Candidatus* (Ca.) *Caldiarchaeum subterraneum* of the proposed Aigarchaeota division (formerly the hot water crenarchaeotic group I) was proposed to be capable of autotrophy in conjunction with H_2 and CO as electron sources and NO_3^- and/or O_2 as electron acceptors (60). Additional genomic comparisons incorporating representatives of the Aigarchaeota division from alkaline hot springs provide further evidence for the capacity of autotrophic metabolism (70), although some members may also be heterotrophic (70, 71), and it is unclear whether their [NiFe]-hydrogenases are responsible for H_2 oxidation or maintenance of cell redox state (71). Likewise, genomic analysis of members of the Hadesarchaea (formerly the South African gold mine euryarchaeotic group) suggests that they are also reliant on H_2 and/or CO as an energy source linked to NO_2^- reduction and capable of autotrophy via the acetyl CoA pathway (61). Lastly, the Bathyarchaeota (formerly the miscellaneous crenarchaeotic group) have received considerable attention because of mesophilic representatives distributed ubiquitously in ocean and freshwater sediments (72, 73).

The presence of protein complements necessary for methanogenesis or methane oxidation (including the methane-activating McrA protein) in the first nearly complete genomes of the Bathyarchaeota hinted at the potential for CH_4 -based metabolism (74). Analyses of other genomes from marine sediments, however, have suggested a role for acetogenesis in supporting these organisms (75). Additionally, a recent analysis of group 1 ANME (ANME-1) suggests that their McrA proteins are involved in the oxidation of short-chain alkanes such as butane (76). The McrA protein sequences of the Bathyarchaeota (and Hadesarchaeota) are more closely related to the McrA of ANME-1, rather than methanogens, suggesting that these organisms may actually be involved in short-chain alkane degradation, rather than methanogenesis. Although the exact role of the Bathyarchaeota in subsurface carbon cycling remains unclear, the capacity for autotrophy and a reliance on H_2 is consistent with other subsurface Archaea. Clearly, further cultivation efforts and physiological-based studies are needed to assess the role of these lineages in light hydrocarbon and H_2 cycling in subsurface environments. Nevertheless, the putative capacity for autotrophy and the use of H_2 and/or CO appear to be common among the Archaea present within high-temperature subsurface environments and are consistent with Gold’s suggestions 25 years ago.

In addition to the aforementioned Archaea, studies over the previous 25 years have documented the prevalence of thermophilic sulfate-reducing bacteria (SRB) as key members of deep, hot terrestrial and marine biospheres (46, 62, 77–79). The discovery and genomic characterization of an autotrophic sulfate-reducing Firmicutes bacterium, *Ca. Desulforudis audaxviator*, has provided valuable insight into life in the deep, hot subsurface. *Ca. D. audaxviator* dominates microbial communities at depths up to 2.8 km in the South African Witwatersrand Basin (46, 77). Genomic reconstruction suggests that oxidation of H_2 and formate coupled

with reduction of SO_4^{2-} is likely to drive inorganic carbon and nitrogen fixation in microbiomes dominated by *Ca. D. audaxviator* (46). The lack of evidence for significant auxotrophy suggests that *Ca. D. audaxviator* contains the genomic machinery necessary for maintaining a self-sustainable deep subsurface ecosystem (46). Although it was originally proposed that *Ca. D. audaxviator* comprises a single-species, self-sufficient microbial community, both earlier 16S rRNA gene surveys (77) and more recent genomic analyses have indicated that additional bacterial taxa are present in the fracture waters and that it is an evolutionarily and ecologically dynamic system with considerable horizontal gene transfer (HGT) and viral infection (66). The genome of *Ca. D. audaxviator* encodes for six and three phylogenetically distinct [FeFe]- and [NiFe]-hydrogenases (31), respectively, further underscoring the importance of H_2 in its metabolism and suggesting an important role for HGT in its evolution. Analyses of high-temperature crustal fluids of the subsurface basement rock in the flanks of the Juan De Fuca Ridge spreading center have also indicated a prevalence of SRA/Bs (78–81), where organisms related to *Ca. D. audaxviator* and other SRA/Bs are dominant. Recent metagenomic analyses of these samples have provided a glimpse into the considerable phylogenetic and metabolic diversity of the deep, hot marine subsurface via reconstructed genomes from metagenomes that have expanded on previous molecular surveys (81).

Although much insight has been gained into the nature of life in the deep, hot biosphere over the previous 25 years since Thomas Gold’s seminal work, there are a number of questions that remain unanswered. For instance, what is the role of syntrophy in sustaining and enabling subsurface microbial ecosystems? Recent analyses of 1.34-km-deep warm fracture fluids from the Witwatersrand Basin suggested a putative role of syntrophic interactions in sustaining a diverse community that is fueled by CH_4 (68). Community-wide trophic interactions are likely integral to the functioning of these isolated systems and warrant further functional-based studies. Another paramount question is whether phylotypes that belong to clades that are distributed in both subsurface and surface environments contain unique physiological adaptations that could provide insight into the selective pressures imposed by subsurface environments and inform their evolutionary history. To phrase this question slightly differently: Is life in the subsurface sufficiently isolated from that of the surface to be considered endemic? Demonstrating endemic subsurface lineages would provide the strongest evidence that the deep subsurface hosts conditions that are not met in near-surface environments and remain distinct enough to prohibit the successful dispersal of subsurface organisms into surface environments or vice versa. Additional insight into this question will better constrain the possibility of life beyond Earth.

Is the Subsurface Microbiome Endemic?

The widespread prevalence of *Ca. D. audaxviator* (46) among several fracture waters in South African gold mines (46, 82) provides the best evidence that life in the subsurface is endemic. A second example is provided by the Henderson group 1 bacterial lineage, distantly related to Nitrospirae, that has only been found (at the species level) in a single >1-km-deep molybdenum mine in Colorado (47). Other *Ca. D. audaxviator* species-level phylotypes have only been found in a deep borehole in Nevada (83). Several studies have identified more distantly related *Ca. Desulforudis*-like phylotypes from both deep terrestrial and marine boreholes that provide further evidence that the genus is generally restricted to subsurface environments (77, 84, 85). Regardless of

the precise geographic distribution of *Ca. D. audaxviator*, their apparent distribution in discrete subsurface habitats, when considered with their physiological capacity for self-sustainment in oligotrophic environments, provide strong evidence that this taxon is endemic.

However, distributions of other subsurface-typical organisms into subsurface-only environments have not generally been observed. For instance, the Bathyarchaeota display significant phylogenetic diversity and are distributed across a wide range of environments that include surface sediments, deep-subsurface environments, and hot springs, among others (72, 73). A recent analysis of the distribution of Bathyarchaeota 16S rRNA gene phylotypes across environment types suggested that there are no phylogenetic subgroups that are uniquely associated with subsurface habitats (73). However, several 16S rRNA gene subgroups were enriched in subsurface habitats, suggesting that there is an evolutionary signal, if not entirely cohesive, of habitat preference among Bathyarchaeota phylotypes (73). In contrast, there is no clear phylogenetic distinction between Aigarchaeota phylotypes (*Ca. Caldiarchaeum subterraneum*) that are found in subsurface environments and those that have been found in surface thermal features such as hot springs (71). Similarly, although certain 16S rRNA gene phylogenetic subgroups of Hadesarchaea phylotypes exhibit enrichment of subsurface-derived sequences, there is not a strong phylogenetic signal delineating subsurface Hadesarchaea from those found in surface thermal features, such as in the Yellowstone National Park hot springs (61).

Available cultivation-independent evidence, compiled over the past 25 years, suggests that, although there may be some organisms that are endemic to the deep subsurface (e.g., *Ca. D. audaxviator* and Henderson group 1), there is evidence that many subsurface-typical taxa are also distributed in near-surface environments, such as hot springs. Indeed, it may be more likely that the prevailing environmental conditions that characterize deep, hot, subsurface environments such as oligotrophy, low nutrient flux, high temperatures, highly reducing conditions, plentiful lithogenic electron donors (i.e., H_2 and CO), and oxidant limitation select for the same taxa in both near-surface and subsurface conditions that exhibit these conditions. Comparisons of genomes reconstructed from subsurface populations and those from near-surface populations are needed to further deconvolute whether specific physiological adaptations differentiate closely related genotypes that are found in both deep- and near-surface environments.

What Are the Roles of Hydrocarbons in Sustaining Subsurface Microbiomes?

A central tenet of Gold's deep, hot biosphere proposal was the role of mantle-derived hydrocarbons in supporting microbial life. Although some laboratory studies have suggested that lighter hydrocarbons such as ethane, propane, and butane can be generated today within the Earth's upper mantle (86, 87), the origin of most hydrocarbons present in the subsurface environment (e.g., in Precambrian continental crustal environments) are not likely to be mantle-derived, as Gold initially proposed (8, 9). However, Gold's ideas have led to substantial interest in determining the origin of subsurface hydrocarbons, and much attention has been directed toward these efforts. In particular, serpentinization of olivine can generate elevated H_2 , and that, when high enough in concentration, may catalyze the production of CH_4 (88, 89), although recent work suggests that the extent of CH_4 production may be contextually dependent on the presence of an H_2 steam phase

(90). Regardless of its source, a reservoir of $3 \times 10^{14} \text{ m}^3$ of gas hydrate, primarily in the form of CH_4 , is thought to exist in the subsurface (91), providing an abundant source of reductant to drive subsurface microbial metabolism.

At the time of Gold's publication, the accepted mechanism for how microorganisms oxidize CH_4 was via aerobic bacterial methanotrophy (92), which would be of limited importance in largely anoxic, high-temperature subsurface environments. Although geochemical, isotopic, and activity data (93–95) hinted at the potential for anaerobic CH_4 oxidation at the time of Gold's publication, it was not until later that the importance and mechanistic details of this process were elucidated. For example, CH_4 is now thought to be oxidized biologically under anoxic conditions via a variety of means that include a near reversal of the methanogenesis pathway (96, 97) with a variety of metal oxidants (98, 99), nitrite dependent oxidation of methane (100), and the addition of fumarate to methane (101).

Although the role of CH_4 in supporting subsurface life is better understood, the anaerobic processes involved in the degradation of hydrocarbons are less well understood. Under oxic conditions, hydrocarbon degradation is carried out through the activity of oxygenases and glycol radical enzymes (102). Comparatively little is known about high-temperature ($>60^\circ\text{C}$) oxidation of hydrocarbons heavier than CH_4 by subsurface microorganisms, particularly under anoxic conditions. However, a thermophilic isolate from the Guaymas basin hydrocarbon seep has been shown to grow on propane and *n*-butane via fumarate addition at 60°C (103). More recently, a mechanism for anaerobic butane oxidation via activation by McrA proteins was demonstrated in thermophilic archaeal *Ca. Syntrophoarchaeum* spp. that grow in consortium with SRB (76). This recent finding represents an exciting new breakthrough in our understanding of high-temperature anaerobic alkane degradation, which could be more widespread than previously appreciated. Thus, based on currently available data, the extent to which microorganisms use or modify hydrocarbons in anoxic, high-temperature environments remains unclear and is ripe for further study.

The Future

Much of the deep, hot biosphere remains to be discovered, including the role of this biota in hydrocarbon production or consumption. Moreover, a number of other outstanding questions concerning the nature of geobiological controls on subsurface habitability remain unanswered. For instance, the nature of microorganisms that mediate water–rock interactions via the consumption or production of reaction products or substrates which then shape the subsurface geochemical landscape and the feedbacks that occur between biological and geological processes necessitates further study. Additionally, the production of high-energy substrates such as H_2 from mechanical weathering processes, such as shearing of silica minerals in bedrock during tectonic activity, has been demonstrated (104), and a similar process has been suggested to be of key importance in supporting hydrogenotrophs in near-surface environments (25). Given the prevalence of silicate minerals in Earth's crust and the prevalence of tectonic activity both today and in Earth's past, it is possible that this mechanism of H_2 production plays a role in supporting a deep, hot biosphere.

The pioneering ideas proposed by Thomas Gold inspired a generation of researchers in the field of geobiology to dive deeper into the possibilities of subsurface life, spawning hundreds of relevant publications (Fig. 1). Importantly, considerable funding

from the National Science Foundation for drilling expeditions over the previous 10 years, in concert with the Integrated Ocean Drilling Program efforts, have led to tremendous new insights in marine subsurface settings. Fifteen years ago, the first drilling expedition that focused on understanding microbial processes in deeply buried marine sediments was conducted, and an increasing number of expeditions with microbial focus have since been undertaken, with several more planned. Indeed, without these funding initiatives, much of the body of research produced since Gold's 1992 paper (Fig. 1) would not have been possible.

Nevertheless, a continuing roadblock to further study and understanding of the deep biosphere is site access, particularly in terrestrial systems. Boreholes are enormously expensive and difficult to obtain. The expense associated with access to subsurface environments and the compelling nature of questions that remain unanswered require a deep subsurface microbiome initiative that aims to synthesize research from marine and terrestrial settings. Such an initiative would provide progress which is greater than that which can be achieved by a single laboratory or a small group of laboratories. For example, an entire field exists to drill such boreholes in the oil and gas industry. If an open collaboration between scientists and the companies that drill ever deeper to extract hydrocarbon resources can occur through a deep subsurface microbiome initiative, a greater understanding of how microorganisms interact with the hydrocarbon-rich settings kilometers below our feet will be unveiled. Moreover, an expanse of underground research laboratories exists for high-energy physics and radioactive waste disposal, in addition to commercial mines and tunneling projects that have not been studied

microbiologically. Such information obtained from expanded studies into these subsurface portals will not only allow us to further our understanding of the limits of life on Earth and address hypotheses put forth by Gold, it will also allow us to refine our predictions for the potential for life on other planetary bodies. Deep hydrocarbon deposits on Mars, Titan, and worlds beyond could play host to life similar to that in Earth's own crust. The techniques used to better study and understand deep, hot biospheres on Earth could then be applied to robotically probe targets in deep space as we move into the next century of scientific discovery. Technology is advancing at a rate wherein we may find that Gold's deep, hot biosphere is not only true, but common across the universe. That would be quite a legacy for a man who routinely thought outside of his own field and thought that he would have been burned at the stake in a different age.

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