18

The Genetics, Biochemistry, and Biophysics of Carbon Cycling by Deep Life

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18.1 Introduction

Much of the microbial life on Earth resides below the surface in the crust (Figure 18.1) (1), either buried in marine sediments (2) and petroleum deposits (3) or entrained in aquifers within oceanic and terrestrial rocks (Figure 18.2) (4–8), fluid inclusions in salt, permafrost, and ice (9–11), as well as hydrothermal and geothermal fluids (12,13). The study of deep subsurface life has defined our understanding of habitability and expanded our knowledge of the mechanisms that enables life to live in these environments (14). While the study of deep life may seem like a philosophical exercise, understanding this enigmatic biosphere has important real-world implications for assessing the safety and feasibility of underground storage of spent nuclear fuel and other toxic compounds, sequestration of atmospheric CO_2, or acquisition of fuels such as tar sands, deep subsurface coal beds, methane hydrates, or fracking (3,5,15).

Organisms inhabiting subsurface environments likely have been isolated from the surface world for hundreds to millions of years (16). Thus, their metabolic lifestyles may differ substantially from those of surface organisms. Even though subsurface environments are diverse (Chapter 16, this volume), subsurface microbes share common biological challenges such as limitations of energy, resources, and space, as well as extremes of pressure, pH, osmolarity, and temperature (Chapter 17, this volume). On the other hand, subsurface environments offer biological advantages, too: environmental stability, protection from UV irradiation, and oxygen. These unique subsurface conditions lead to communities that are often phylogenetically and functionally diverse, with extremely slow population turnover times (14,17,18) and efficient energy metabolisms (14,19). Increasingly, the roles of viruses and eukaryotes, in addition to bacteria and archaea, are being recognized in the deep subsurface biosphere (20–25). Several barriers hamper the study of life in Earth's crust, such as sample acquisition and the difficulty of retrieving sterile, unaltered samples that have not been contaminated by drilling fluid. However, an even bigger hurdle is the difficulty of studying the copious subsurface microbes with no cultured representatives (13,26). Their functional potential must be pieced together from direct assessments of biomolecules or biochemical processes in natural samples. However, even subsurface microbes related to laboratory cultures with "known" functions, may not perform those functions in the natural
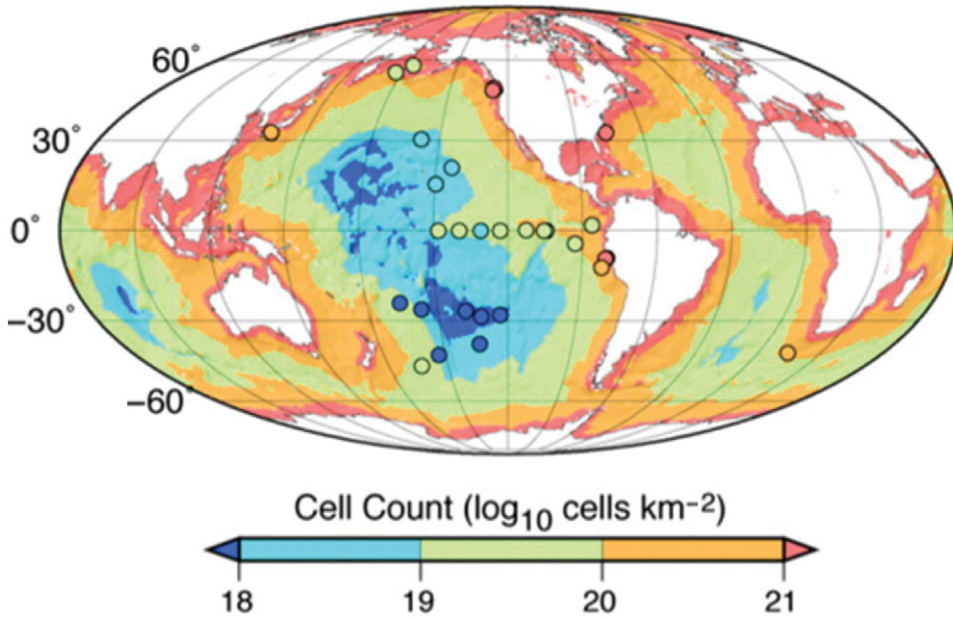
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Figure 18.1 Global distribution of marine sediment densities of microbial cells, integrated with depth (adapted from 1).
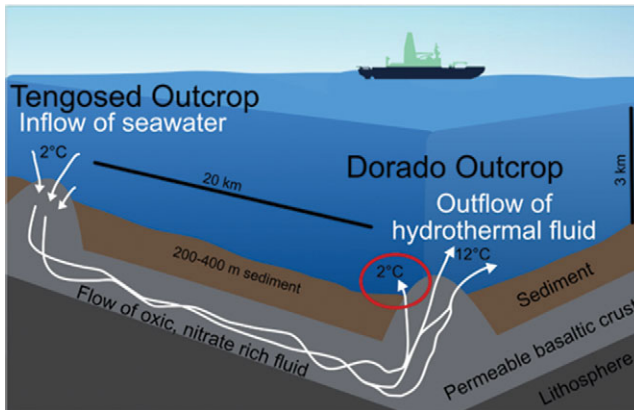

Figure 18.2 Example schematic of a deep subsurface basaltic aquifer. Such environments provide a substantially different habitat for microbial life from sediments or soils (adapted from 33).

environment. Nonetheless, culturing has been extremely important in establishing the existence of a living deep subsurface biosphere (27–32). Researchers must therefore combine direct molecular biological assessments with geochemical and geophysical environmental parameters to describe how carbon is microbially transformed in subsurface environments.

18.2 Genetic Potential of Subsurface Environments

Much research addresses the question of how subsurface life differs from surface life in genetics, biochemistry, and biophysics. Culturing-based methods are still the cornerstone of microbiology because they enable physiological assessments in carefully controlled experiments. However, it is universally accepted that culturing-based methods typically recover a very small proportion of the total microbes from any environment (34–36). Our inability to cultivate microbes is a multifaceted problem that in part has been driven by our inability to perfectly recreate the physical and chemical conditions necessary for growth, slow microbial growth rates, and the rise in popularity of molecular techniques that produce data rapidly. Thus, like the rock record, our views of microbial life and the processes they perform *in situ* are incomplete. While this may seem bleak, there is renewed interest in the cultivation and development of novel culturing techniques, such as high-pressure culture vessels, and this is being spurred, in part, by discoveries made through genome sequencing.

Ribosomal gene sequencing has revolutionized our understanding of the microbial evolution and diversity of microbial phyla (37–39). These culture-independent methods have been a boon for the study of subsurface life, as many of the organisms in the subsurface require direct environmental sequencing because they are slow growing (40), potentially dormant (41,42), or require specific growth conditions or partners (43). Single-gene amplicon sequencing (i.e. 16S rRNA or nitrate reduction genes) remains the most used method for assessing microbial communities, but it lacks the resolution to decipher the metabolic potential of the microbes observed. However, other popular sequencing-based methods can also indicate metabolism, like single-cell amplified genomes (SAGs) or metagenomics and metatranscriptomics, where total community DNA and RNA are sequenced directly from environmental samples (4,44,45). The Census of Deep Life (https://vamps2.mbl.edu/portals/CODL), within the Deep Carbon Observatory, has enabled DNA sequencing from many deep subsurface environments and will provide a great resource as this work continues (46).

The main hurdle for the molecular-based analysis of subsurface environments is the amount of microbial biomass present in the sample, which is often low. Nucleic acid extraction from any environmental sample is a fundamental step for all molecular-based studies (Figure 18.3). Extraction methods are extremely diverse and highly dependent on the samples being processed. Recent work has highlighted that commercial DNA/RNA extraction kit reagents, typically designed for high-biomass environments, contain their own microbiome that can skew downstream amplicon data analyses (46,47). It is also important to remove reagent contamination from SAGs, metagenomes, and metatranscriptomes (48–50). Thus, great care must be taken to screen resulting reads and assembled contigs to identify and remove suspicious sequences, while also allowing that sometimes organisms that were previously thought to represent surface contamination, such as cyanobacteria, may actually be legitimate community members (51).

Post-sequencing analysis of genomes, metagenomes, and metatranscriptomes has made significant advances that have greatly enhanced our knowledge of subsurface life (52–54).
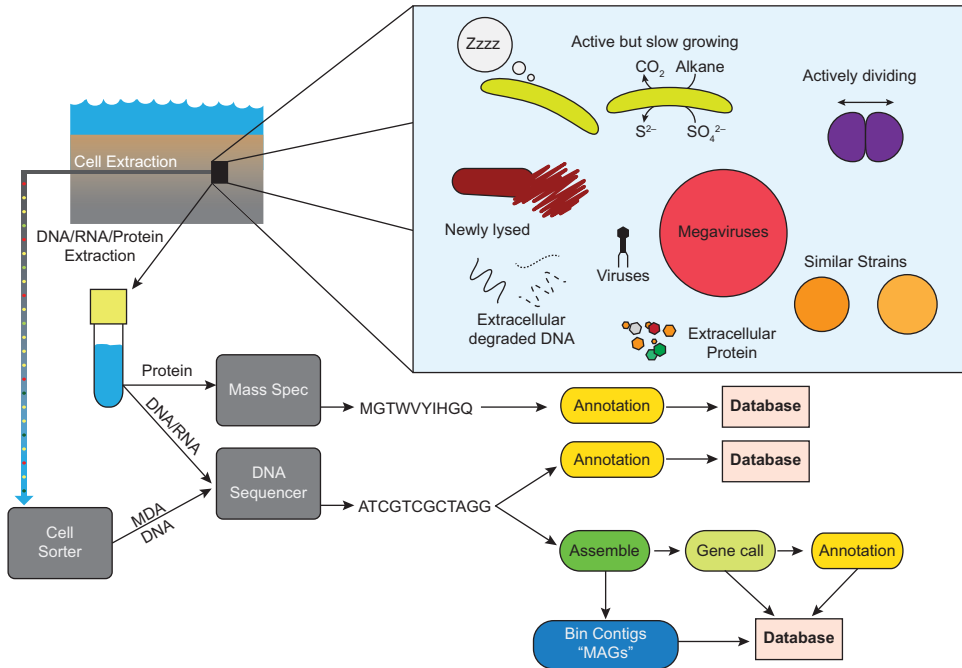
Figure 18.3 Schematic workflow for molecular studies of subsurface ecosystems. MAG = metagenome-assembled genome; MDA = multiple displacement amplification.

The ability to identify and extract microbial genomes from metagenomes (i.e. genome binning, also referred to as metagenome-assembled genomes (MAGs)) (55), along with SAGs, has enabled physiological inferences to be made for uncultured subsurface organisms. Recovery of genomes from metagenomes like *Candidatus* Desulforudis audaxviator (52) or *Leptospirillium* and *Ferroplasma* (56) revealed that microbes living in low-diversity, subsurface environments contain a large amount of genomic diversity, strain heterogeneity, and physiological diversity. *Ca.* D. audaxviator and *Ca.* Desulfopertinax cowenii contain metabolic pathways for heterotrophic growth as well as the potential to fix carbon via the Wood–Ljungdahl (WL) pathway (57). In subsurface environments, the WL pathway has been increasingly identified in MAGs and SAGs. The presence of the WL pathway is intuitive as it requires minimal energy to fix C to biomass (58) and is reversible to oxidize acetate. Interestingly, energetically taxing pathways such as the Calvin–Benson–Bassham cycle are also present (4,59). Metagenome sequencing and subsequent genome binning have been applied to several subsurface environments, such as sediments (marine and freshwater), aquifers, rock, caves, mines, and marine basalts (4,60–68). In these systems, chemolithoautotrophy is always counterbalanced by heterotrophs and fermentative organisms (4,61). As more subsurface environments are sampled and sequenced and their genomes assembled from metagenomes, researchers have the ability to apply

pangenomic and phylogenomic approaches (69) to look for functional and evolutionary differences in subsurface populations. Jungbluth et al. (57) applied these techniques to show genomically that deep terrestrial subsurface *Ca.* D. audaxviator lacks carbon uptake genes that the marine subsurface *Ca.* D. cowenii has, indicating the potential for metabolic plasticity. SAGs have also been used to target and recover specific genomes of interest from the environment (22,70,71). These targeted approaches provided insight into the carbon metabolism of two ubiquitous and enigmatic Archaea (72). However, as with metagenome sequencing, recovery of complete genomes can be difficult (48). Regardless of method, these tools have provided an unprecedented glimpse into the diversity – in terms of the number of both microbes and functional genes – of subsurface microbes, and they finally allow microbiologists to draw conclusions as to the functional interactions between microbes that drive system productivity (4,60,64,66).

Metatranscriptomics provides a snapshot of the environmental conditions microbes sense at the time of sampling, as the turnover of RNA in the cell is rapid, being in the order of minutes for many microbes (73). Orsi et al. applied a metatranscriptomic-based approach to show that sub-seafloor sediment microbes express genes for carbohydrate and protein degradation pathways (45). Lau et al. (43) were able to construct metabolic interaction maps of microbial communities in a terrestrial subsurface South African gold mine. These microbial communities appear to use a diversity of carbon fixation and decomposition pathways to cycle carbon. *De novo* assembled metatranscriptomes can be a useful tool for looking at broad patterns of expression in the subsurface (43,74) or the contribution of rare community members to biogeochemical cycles (75). However, this method is highly dependent on the annotation databases and thus could missannotate functions or be unable to give a phylogeny. Even with these caveats, metatranscriptomics coupled to either MAGs or SAGs can link potentially novel microbes to biogeochemical cycles.

Genes and gene transcripts do not always link directly to organismal activity due to post-transcriptional regulation of microbial functions (76). Therefore, proteomics may be a way to probe microbial functions of the entire community more directly. This method has only recently been applied to deep subsurface samples (43). Lau et al. showed that a wide variety of metabolic activities co-occurred at a 1.34-km deep fault zone, implying that the organisms acted syntrophically to support an autotrophic-based ecosystem (43). Proteomics and genomics also suggested metabolic interdependencies among subsurface microbes in an acetate-fed aquifer (77). Another method that pushes closer to measuring microbial activity *in situ* is metabolomics, which uses mass spectrometry to identify all of the small organic molecules present in a sample. This, too, has the potential to point to metabolic pathways that are functional in deep subsurface samples, but it has only recently come into use (78). The greatest challenge facing metabolomics is dealing with the large number of unknown structures in the data set – a problem shared with all complex microbial communities.

The rapid acceleration of data produced from genomic, transcriptomic, and soon proteomic and metabolomic research into deep subsurface environments has opened up a new dimension regarding how we learn about this difficult-to-culture biosphere. Currently,

data production far outstrips the ability of a single research lab to analyze each data set to completion. This is already beginning to support a potentially rich research area of meta-analysis studies, where combining data sets from multiple studies allows for novel insights not apparent from a single data set alone (e.g. 46). Through further pushing of the boundaries of what can be achieved by applying biomolecular tools to environmental samples, there is no limit to what researchers can learn about this important biosphere. Such studies not only overcome some of the limitations of whole communities full of uncultured clades, but will also lead researchers to new approaches to achieve culturing of some of these recalcitrant growers.

18.3 Biogeochemistry of Deep Subsurface Life

Microbes are Earth's most prolific chemists. They do this by transforming organic and inorganic elements and compounds using enzymatic protein complexes. Earth's rock record provides a glimpse of some of these processes and their evolution over time, either through isotope signatures or mineral formations, with evidence for the coevolution of minerals and biology (79,80). While the rock record provides a hint of what processes were in operation when they were deposited, interpreting these observations relies on the characterization of extant microbes.

Microbes transform the carbon landscape of Earth's subsurface largely by "eating" or "breathing" carbon-containing compounds (81). Respiring microbes use one molecule as an electron source (reductant) and another molecule as an electron sink (oxidant). Common reductants are organic molecules such as carbohydrates, amino acids, lipids, or small molecules like hydrocarbons, acetate, and formate, as well as inorganic compounds such as reduced sulfur, ammonium, transition metals, or H_2. Carbon-containing oxidants include CO_2 (in the case of methanogenesis), organic molecules like fumarate or oxalate, as well as inorganic compounds like O_2, oxidized sulfur or nitrogen compounds, or oxidized transition metals. An alternative to respiration is fermentation, where microbes split a single molecule of intermediate oxidation state into a more oxidized one and a more reduced one. The reduced product often serves as the reductant for another organism's respiration. Organic matter is the most common fermentative substrate, but microbes can also ferment elemental sulfur through disproportionation. In addition to respiration and fermentation, which are primarily performed to give a microbe energy, microbes can also expend energy to take up carbon compounds. Most notably, primary producers incorporate CO_2 to build biomass. Therefore, every type of microbe transforms carbon, and the presence of life on Earth has had profound effects on its carbon cycle.

To describe transformations of carbon compounds in deep subsurface environments, one must determine what combination of reductants, oxidants, and/or fermentative substrates are utilized, which microbial communities use them, and how quickly they do so (14). In many deep subsurface environments, highly oxidized oxidants such as oxygen and nitrate have already been consumed by shallow organisms, leaving the less powerful (per mole) oxidants such as sulfate and CO_2 to dominate (82,83). However, exceptions occur in

environments with conduits connecting to surface-derived fluids such as deep basalts or surface-connected aquifers. In areas of the ocean floor with extremely slow sedimentation rates, O_2 can be detected many meters deep into marine sediments (33,84–86). CO_2 fixation, usually powered by chemolithoautotrophy rather than photosynthesis, is a ubiquitous and important carbon transformation process in deep subsurface environments (87).

Through the application of novel biomolecular techniques that complement the DNA, RNA, and protein-based studies descried above, great strides have been made in the understanding of the biochemistry of microbes performing carbon transformation in Earth's subsurface. These methods include enzyme assays, lipid analysis, stable isotope probing (SIP), metabolomics of small metabolic intermediates, measuring or modeling respiration rates, and heterologous expression of novel enzymes from deep subsurface organisms.

18.3.1 Microbial Metabolism in the Deep Subsurface

Measuring the rate that a microbial function occurs is essential for determining how the function contributes to the carbon cycle. Older methods to study microbial activity in the deep biosphere, such as the incorporation of radioactive thymidine into bulk samples, have largely fallen out of favor since they are insufficiently sensitive to detect microbial activities below a few meters deep into sediments (88). A more sensitive method for estimating *in situ* microbial activity is to measure the turnover rate of trace amounts of radioactive nutrients such as electron donors/acceptors. Such methods have attained widespread use in subsurface environments and have shown that SO_4^{2-} and CO_2 serve as important electron acceptors for deep subsurface biosphere respiration (89). In agreement with this, DNA signatures for microbes capable of these types of respiration have been identified in many subsurface locations (e.g. 90,91). Although it may seem that better oxidants should correspond to faster organic matter degradation, recent work has suggested that the rate of carbon remineralization operates independently of which oxidant is available (92). Here, radiotracer-measured rates of carbon oxidation to CO_2 were constant, whether sulfate or CO_2 was the terminal electron acceptor.

Direct microscopic assessment is often uninformative about either taxonomy or physiology because cells tend to be small (93,94) and their morphology does not indicate their identity or environmental functions (2). In recent years, SIP coupled to fluorescence *in situ* hybridization (FISH) and nanoscale secondary ion mass spectrometry (NanoSIMS) has enabled the direct metabolic assessment of natural populations incubated *ex situ*, but still within their natural microbial community. SIP works on the principle that organisms taking up isotopically labeled substrate will retain it in their biomolecules and can later be identified. After 9 and 405 days of seafloor incubation of a sediment core with ^{13}C-labeled glucose, Takano et al. (95) found that subsurface archaea incorporate fresh glucose in the glycerol headgroups of their membrane lipids, but rely on detrital carbon recycling for the hydrocarbon isoprenoid groups of the lipids. FISH allows the microscopic identification of microbes of a particular taxonomic group by attaching fluorescent probes to short DNA

sequences that match the taxonomically informative ribosomal RNA present in cells (96). In places with low activity, such as the deep subsurface, the dim fluorescence signals of FISH often require amplification with catalyzed reporter deposition (CARD) (97). Nano-SIMS sputters a cesium ion beam across an area of a few micrometers to detect any isotopic label taken up by the microbe during the SIP incubation. Therefore, when combined with FISH, NanoSIMS SIP gives information about what substrates were consumed by which taxonomic groups of organisms on a nearly single-cell basis.

In 2011, Morono et al. used NanoSIMS SIP with CARD-FISH to show that cells in up to 460,000-year-old sediments, ~200 m deep into the sub-seafloor, were capable of growing on glucose, pyruvate, and amino acids (98). In addition, acetate and bicarbonate were incorporated into the biomass, although they did not promote growth. However, whether the identity of the cells that grew in these experiments reflected the community present at the time of sampling was not assessed, since only a subset of the growing cells hybridized to FISH probes (98). This could be the result of either mismatches to the probe DNA sequences or difficulties of hybridizing cells during CARD-FISH (99,100).

In one of the deepest sub-seafloor drilling operations to date, researchers on Integrated Ocean Drilling Program (IODP) Expedition 337 found relatively high numbers of microbial cells whose taxonomic identities resembled terrestrial communities in 2-km-deep coal beds, suggesting that these cells had persisted through the burial of these terrestrial sediments over tens of millions of years (101). By combining SIP, CARD-FISH, and NanoSIMS, Trembath-Reichert et al. found that these communities incorporated methylated compounds into the biomass, sometimes with concomitant methane production (102). Ijiri et al. combined clumped isotopic measurements and label turnover rates to show that deep fluids stimulate substantial methanogenesis at mud volcanoes (103).

An analogous method to SIP that allows single-cell analysis without requiring an expensive NanoSIMS instrument is called bio-orthogonal noncanonical amino acid tagging (BONCAT) (104). In this method, natural samples are incubated with an amino acid mimic equipped with a functional moiety that can react with a fluorophore after the incubation has ended. In this way, the microbial cells that were making new proteins (and therefore incorporated the amino acid mimic) fluoresce under a microscope and can be counted by eye or physically separated in a flow cytometer for downstream biomolecular study. BONCAT was used in subsurface samples to show stimulation of amino acid incorporation in microbial aggregates incubated with methane (105).

Metabolism can also be controlled by entering an inactive or dormant state. Among cultured bacteria, the Firmicutes and Actinobacteria are capable of forming endospores that can withstand low nutrients and inhospitable conditions for long time periods. These properties make sporulation a potentially advantageous phenomenon for deep subsurface life; however, it is possible that common DNA analyses miss spores since they can be difficult to lyse. Therefore, Lomstein et al. measured dipicolinic acid, a key spore coat protein, in the deep subsurface of the Peru Margin to estimate that spores in this deep subsurface environment may be as numerous as nonsporulated cells and the necromass from other dead cells that may provide food for metabolically active microbes (40).

However, when the decrease in cellular biomass over sediment age is instead used to estimate the microbial necromass production rates, the rate is far outstripped by organic matter buried from ancient pelagic sedimentation (106).

In marine sediments with low organic matter contents and deep terrestrial aquifers, the radiolysis of water may provide microbial food. Natural radioactive decay of Earth's minerals can slowly split water into H_2 and oxidized moieties (107). Hydrogen production from Earth's background radiation may seem inconsequential relative to the power provided by sunlight in surface environments. However, H_2 production rates from water radiolysis have been shown to be sufficient to support life in the deep subsurface (52,107,108). The deep subsurface may have additional sources of energy such as serpentinization, which produces the microbial substrate H_2 and has been shown to support many microbial communities (109,110). Serpentinization also produces a large amount of alkalinity, which inhibits autotrophy by pushing carbonate equilibrium away from CO_2. Therefore, unique types of methanogens have been discovered in serpentinizing systems that have novel mechanisms for ameliorating CO_2 limitation (111,112).

A feature that may be very important to subsurface ecosystems is symbiotic relationships. Historically, microbial communities from deep subsurface aquifers have been studied by examining the cells that are caught on a 1.2-μm filter, but recent work has shown that a wide range of ultrasmall bacteria pass through these filters (4). These cells form previously undiscovered branches on the tree of life called candidate phyla radiations (113). In addition to having extremely small cell sizes (0.009 ± 0.002 μm^3), they have very small genome sizes as well, and they lack many features thought to be important to free-living organisms (4). In addition, their cell structures show that they have cellular appendages that might aid in cell-to-cell communication, similar to those of ultrasmall uncultured archaea called archaeal Richmond Mine acidophilic nanoorganisms (ARMAN) (Figure 18.4) (94). Therefore, these candidate phyla radiations have been proposed to be obligate symbionts of larger microbes (4).

18.3.2 Predicting Functions of Novel Genes

Many of these genes in subsurface organisms cannot be accurately annotated by their homology to characterized gene products, since most of the latter come from microbes in pure culture (114). Given that genomic data are quickly becoming the most accessible type of data to shed light on deep biosphere communities (Section 18.2), the inability to interpret the functions of much of their genomes is a major limitation. For instance, from its genome and phylogeny, *Desulfurivibrio* sp. appear to be anaerobic sulfate reducers. However, when they were obtained in pure culture, they did not reduce sulfate, but instead oxidized it (115). A genome from an uncultured phylum contained a homolog of the methyl coenzyme M reductase gene, from which one might conclude that it performs methanogenesis or anaerobic methane oxidation. However, enrichments of this organism could not perform methane metabolisms, but instead oxidized butyrate anaerobically (116).

One method for overcoming difficulties in accurate annotations of genes in organisms that cannot undergo phenotypic ground-truthing is to amplify target genes of interest
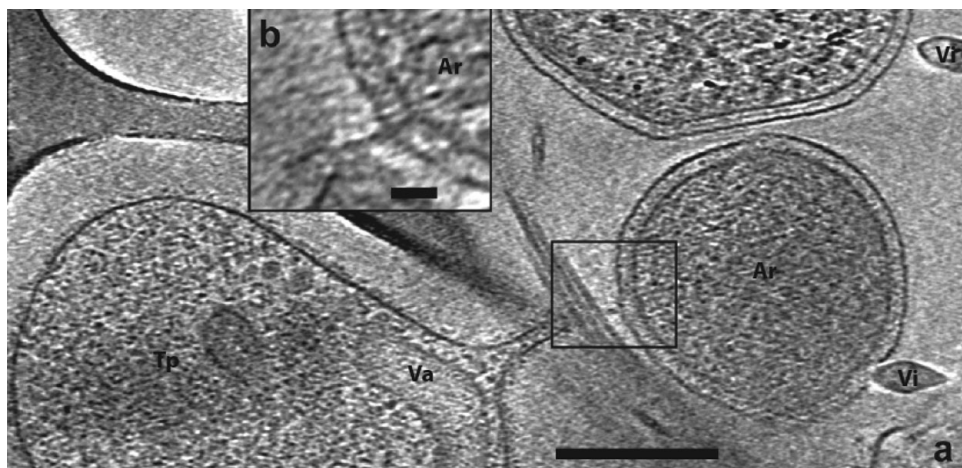
Figure 18.4 Cryo-electron tomographic reconstruction of ARMAN archaea (Ar), Thermoplasmatales (Tp), vacuole (Va), and virus (Vi). (a) shows the larger image, with a 300-nm scale bar, and (b) shows the inset, with a 50-nm scale bar.
Image reproduced from (94).

directly from natural samples or single-cell genomes, ligate them into DNA plasmid vectors, transform them into *Escherichia coli*, express them as proteins, purify them, solve their structures, and characterize their activities. A predicted gene annotated as a peptidase in the uncultured phylum Bathyarchaeota was heterologously expressed in *E. coli* and crystallized in order to determine its structure and catalytic activity (117). Although the gene was annotated as an S15 peptidase with substrate specificity for terminal proline residues, the expressed enzyme, called bathyaminopeptidase, was found to have a much higher affinity for terminal cysteine residues. This distinction illustrates the limitation of inferring enzyme function from homology to known proteins in these uncultured deep subsurface microbes. Homologs of the gene for the II/III form of Rubisco, a well-studied key gene for CO_2 fixation in the Calvin–Benson–Bassham cycle, occur in the candidate phyla radiation among the bacteria in deep terrestrial aquifers. This enzyme had previously only been found in cultured archaea, so its presence in uncultured phyla of deep subsurface bacteria suggested that perhaps it had different catalytic properties in these unknown groups (4,118). However, adding the Rubisco II/III gene from the candidate phyla radiation lineage to a phototroph, *Rhodobacter capsulatus*, which had been engineered to remove its native Rubisco gene and had therefore lost its CO_2 fixation capabilities (119), caused its full CO_2 fixation capabilities to be revived (59).

The limitation of this heterologous gene expression approach is that it has much slower throughput than genetic characterizations. However, it provides hard evidence for function that is lacking when simply inferring enzymatic functions from homology to known proteins. In addition, it presents opportunities for discovering novel functions rather than making assumptions based on previously characterized proteins.

18.3.3 Cellular Bioenergetics

Overcoming energy limitation is a major focus of life in both marine and terrestrial subsurface environments (11,14). Reaction transport modeling of oxygen and nitrate concentration profiles with sediment age showed that deep subsurface communities in the South Pacific Gyre operate at extraordinarily low metabolic rates (85,86). Subsurface microbes are therefore operating in a low energy state, and the majority of these populations may be in a long-term stationary stage in which they require energy only for cell maintenance (120,121). Determining the minimum value for cellular maintenance is difficult, but nanocalorimetry, in which the kinetics and thermodynamics of very small populations of cells can be measured (122), and long incubations with no new substrate additions (123–125) hold promise for studying organisms operating at very low energies. At the very minimum, living cells must replace biomass fast enough to overcome abiotic racemization rates, which can be very slow (126,127). Determining true growth rates for such natural microbial populations outside of carefully controlled laboratory conditions is nearly impossible because the change in the number of cells over time represents the product of growth rate minus death rate. However, total population turnover rates can be determined (14). Braun et al. used racemization rates to estimate total population turnover times of up to tens of years for marine sediment microbes in relatively organic-rich parts of the ocean (17). Slow biomass turnover times of several months to over 100 years have also been measured by measuring uptake of deuterated water (Figure 18.5) (102).
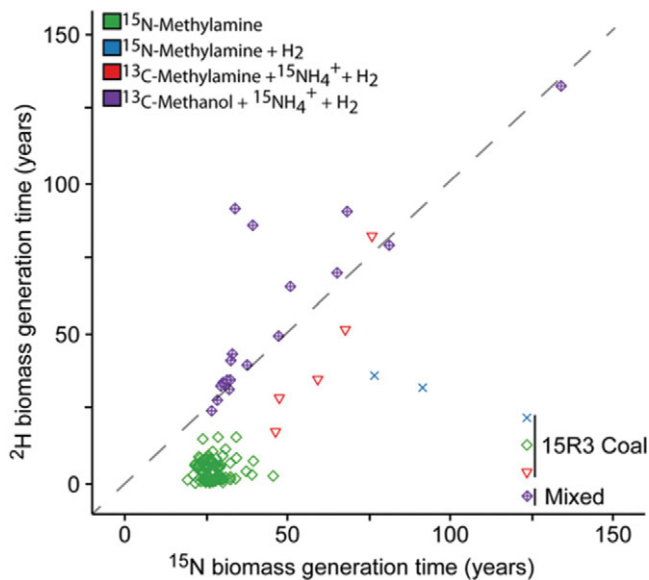

Figure 18.5 Generation times for microbial life in 2-km-deep sub-seafloor coal and shale beds based on uptake rate of either ^{2}H-labeled water or ^{15}N-labeled compounds as described in the graph's key (adapted from 102).

One challenge to such research is the difficulty in making accurate absolute quantifications of specific microbial populations, not just total cell counts, in natural samples that can have very low biomasses or features that disrupt quantification methods. For instance, marine sediments themselves can obscure quantifications with FISH and quantitative polymerase chain reaction (99), and extremely small cells might be missed during filtration of water samples (94). However, a method that accurately quantifies the absolute cellular abundance of particular taxonomic groups in natural deep subsurface samples has yet to be developed.

18.4 Pressure Effects

All lifeforms depend on the chemistry and physics of self-replicating and self-assembling macromolecules, such as proteins, to catalyze the chemical reactions described above. The goal of research in extreme biophysics is to understand how extreme physical conditions affect the fundamental processes responsible for sustaining life. This includes understanding mechanisms of molecular adaptation to extreme conditions and the driving forces of evolution throughout geologic ages.

Much of life on Earth exists under what are considered extreme conditions of pressure: 88% of the volume of the oceans is at an average pressure of 38 MPa, the deepest trenches can exceed 100 MPa, and microbes exist at pressures as much as threefold higher deep in Earth's crust (128). The biomass in high-pressure environments may exceed that on the surface (128). In the oceans, temperature decreases with depth until it reaches an almost constant $3°C$ with pressure as high as 1000 atmospheres (128). Most of the high-pressure ocean is cold and dark, with minimal sources of carbon and minerals and with a low but constant concentration of oxygen. In contrast, temperatures of the water exiting hydrothermal vents in the deep oceans can reach $400°C$, with a temperature gradient so large that the temperature decreases to $3°C$ only 1 m from the vent. This large temperature gradient on the ocean's floor may be partly responsible for the highly diversified and dynamic deep subsurface microbiome. In the deep subcontinental crust, the total volume in the hydrated fissures in the rock is a large proportion of the biosphere, estimated to be 1016 m^3 and 23–31 Pg of carbon (128,129). If only 1% of that volume is occupied by microorganisms, their biological productivity could be greater than that of Earth's surface (128). It is truly remarkable that life manages to exist in such extreme environments compared to the benign conditions in which humans exist.

18.4.1 Extreme Molecular Biophysics

The structures, interactions, and functions of biological macromolecules are highly sensitive to temperature, pressure, pH, salinity, and the concentration of small osmolytes. Take, for example, proton/sodium-coupled electron transfer (PCET) processes, which are central to energy production. The molecular machinery for H^+-coupled ATP synthesis is

essentially the same across most living cells; ATP synthesis succeeds regardless of physical conditions, even though the chemistry of H^+-driven processes in biological macromolecules is highly sensitive to pressure, temperature, and salinity. The adaptive mechanisms for PCET under extreme conditions are unknown. Likewise, the physical properties of both the proteins and nucleic acids that drive the central dogma are highly sensitive to temperature, pressure, pH, and salinity, and yet DNA transcription and translation succeed even in extreme environments. The importance of understanding the molecular mechanisms of adaptation of fundamental biochemical processes to extreme conditions cannot be overstated. Somehow, in these extreme environments, adaptations take place so that the essential processes necessary to sustain life can proceed unimpeded.

Whereas structure–function–thermodynamics relationships in proteins from thermophiles have been studied extensively, studies on the adaptation to high pressure are limited (130). Notable exceptions are pressure-adapted RNA polymerases (131), metabolic enzymes (132–138), the bacterial chromosome organizer, histone-like nucleoid structuring proteins (139), G-protein-coupled receptors in deep-sea fish (140,141), and single-stranded DNA binding proteins (142). In addition to identifiable adaptations at the level of the protein's primary sequence, pressure adaptation at the cellular level has been shown to involve changes in gene expression (143), such as upregulation of specific transport proteins of the outer membrane protein (OMP) family (144,145) and the production of osmolytes (146–149), chaperones (150,151), polyamines (152), and modified lipids (149,150,153–155) (Figure 18.6).

Biophysical aspects of adaptation to extreme environments are poorly understood, although the effects of extreme conditions on mesophilic organisms have been studied extensively. Pressure can modulate the conformation of lipid bilayers, nucleic acids, and proteins (156). In the case of nucleic acids, structures can be either stabilized or destabilized by pressure (157), although in general, RNA and DNA molecules exhibiting tertiary structures, such as hairpins, quadruplexes, and ribozymes, are disrupted by pressure (158,159). Lipid bilayers are compressed and thickened in response to pressure and become much less dynamic. The melting temperature for gel to liquid crystalline transitions increases with pressure, and organisms living at high pressures are known to have a higher proportion of unsaturated lipids in their bilayers to maintain an appropriate degree of fluidity.
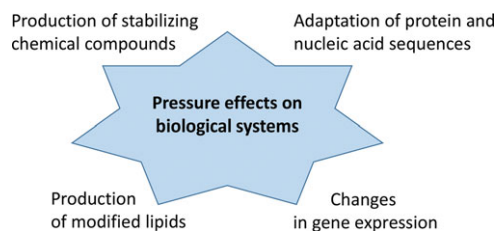

Figure 18.6 Mechanisms of pressure adaptation.

It has long been known that hydrostatic pressure leads to protein unfolding (160) and oligomer dissociation (161,162). Pressure effects on protein structure arise from differences in molar volume between different conformational states. The molar volume of the unfolded states of proteins (U) is generally smaller than that of their folded states (F) (Figure 18.7), as is the molar volume of monomeric subunits compared to oligomeric species. Pressure shifts the folding/association equilibria toward the state that occupies the lowest molar volume: the unfolded or dissociated states. The values of these volume changes are quite small, typically <150 mL/mol for the unfolding of small globular proteins and <300 mL/mol for the dissociation of oligomers.

Although the differences in volume between conformational or oligomeric states of proteins have been well known for some time (165,166), their structural origins remained obscure and were hotly debated until recently. It has been shown repeatedly that substitution of a large hydrophobic amino acid side chain such as leucine, isoleucine, or even valine by a smaller one such as alanine or glycine invariably leads to a larger overall magnitude of the volume change of unfolding and to greater sensitivity to pressure (167–169). A very strong correlation exists between the volume change of unfolding and the packing density (fractional void volume) of a given protein.
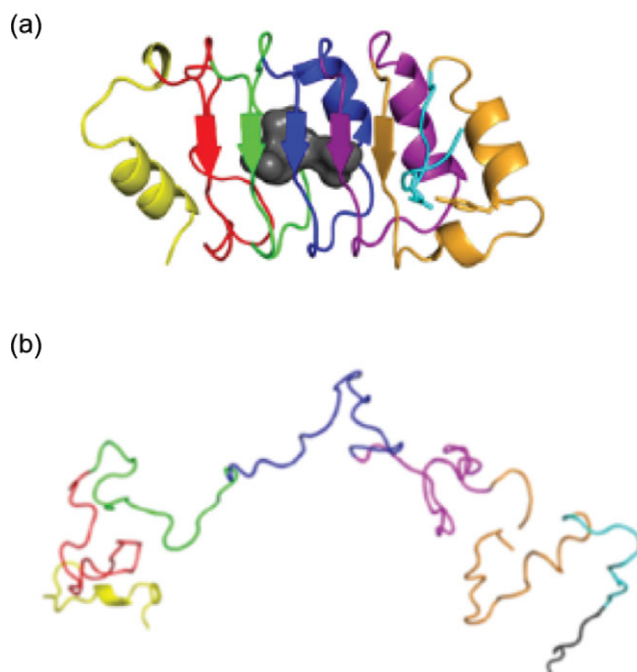
(a)

(b)

Figure 18.7 Schematic diagrams of a folded (a) and unfolded protein (b), in this case the pp32 leucine-rich repeat protein
(adapted from 163). Cavities in (a) were calculated with a 1.4-Å sphere using *HOLLOW* (164).

The destabilization of any given protein by pressure is larger at low temperatures. Chen and Makhatadze proposed that hydration not only does not cause pressure-induced protein unfolding, but actually makes a strong contribution that opposes pressure-induced unfolding (170). However, as temperature increases, the increase in internal void volume becomes smaller than the effects of hydration, and thus the compensation effect diminishes and the overall difference in molar volume between folded and unfolded states diminishes (171), and can even change sign.

Many aspects of the thermodynamic and structural pressure effects on proteins and adaptations to high-pressure environments remain unknown. It was recently shown that there is no pressure-specific "piezolytes" (i.e. there are no specific volumetric effects associated with low-molecular-weight osmolytes). Rather, their effect is simply to stabilize folded proteins against all manner of perturbations, not specifically high hydrostatic pressure (172). It is not well understood whether there are inherent, specific physical attributes of proteins and nucleic acids that allow them to tolerate high-pressure environments. On a larger scale, the ensemble of different organisms in a given environment may modulate biomolecular adaptation through the physical properties of the biomolecules. Large-scale structural adaptive differences may even be identifiable through comparison of whole genomes.

The populations of folding intermediates and low-lying alternative states implicated in the function, degradation, or aggregation of proteins from piezophiles may be different from those of their mesophilic homologs (173,174), Thus, proteins from piezophiles may have evolved to modulate the population of such species.

Thankfully, through sustained progress in instrumentation for fundamental physico-chemical studies of the effects of pressure on biological molecules, the biophysics community is well positioned to address outstanding issues in biomolecular adaptation. High-pressure nuclear magnetic resonance (NMR) measurements, once restricted to a few centers with specialized equipment (175–178), have been adapted for low cost and ease of use with high-field NMR spectrometers (179,180). High-pressure small-angle X-ray and neutron scattering devices are available at several facilities in Japan (181), Europe (182), and in the United States (169). Fluorescence, Fourier-transform infrared spectroscopy, and ultraviolet–visible spectroscopy have long been used in high-pressure biophysical research, and electron paramagnetic resonance and circular dichroism spectroscopy have recently been adapted (183,184). Similarly, high-precision modern microcalorimeters are commercially available, as are electronically controlled, temperature-regulated cells for optical spectroscopic equipment. The range of temperature–pressure studies of biological macromolecules that are possible is broad.

18.4.2 Extreme Cellular Biophysics

In the deep-sea bacterium *Photobacterium profundum* (145), pressure affected the expression of many genes that code for proteins implicated in nutrient transport, such as outer membrane proteins (Omps). In the yeast *Saccharomyces cerevisiae* (185), pressure affected

the expression of heat-shock and metabolic proteins. Upregulation of heat-shock and cold-shock protein expression in *E. coli* in response to high pressure has also been reported (186). It has also been shown that pressure shock of around 1000 bar leads to the filamentation of *E. coli* (187), and 40 MPa has the same effect on piezophilic *Desulfovibrio indonesiensis* (188), although there are likely multiple mechanisms by which this occurs. Pressure may lead to the dissociation of FtsZ, a tubulin-type GTPase involved in forming the bacterial septum for cell division (189). However, certain strains of *E. coli* bear a pressure-activated Type IV restriction endonuclease, Mrr, which leads to a pressure-dependent SOS response and subsequent filamentation (190,191). The molecular mechanism of the pressure-induced SOS response was shown to be due to pressure-induced dissociation of the inactive Mrr tetramer to an active Mrr dimer that cleaves cryptic sites on the *E. coli* chromosome (Figure 18.8).

Although genetics, genomics, and transcriptomics can provide an overview of the putative mechanisms of adaptation to life under extreme pressures, these studies provide little biophysical insight. The study of extreme conditions on living cells remains a daunting technological challenge and requires the implementation of high-pressure quantitative microscopy approaches. A few high-pressure microscope chambers have been developed, although they have not been widely used. In the 1970s, Salmon and coworkers developed a phase contrast microscope that could withstand about 800 bar pressure
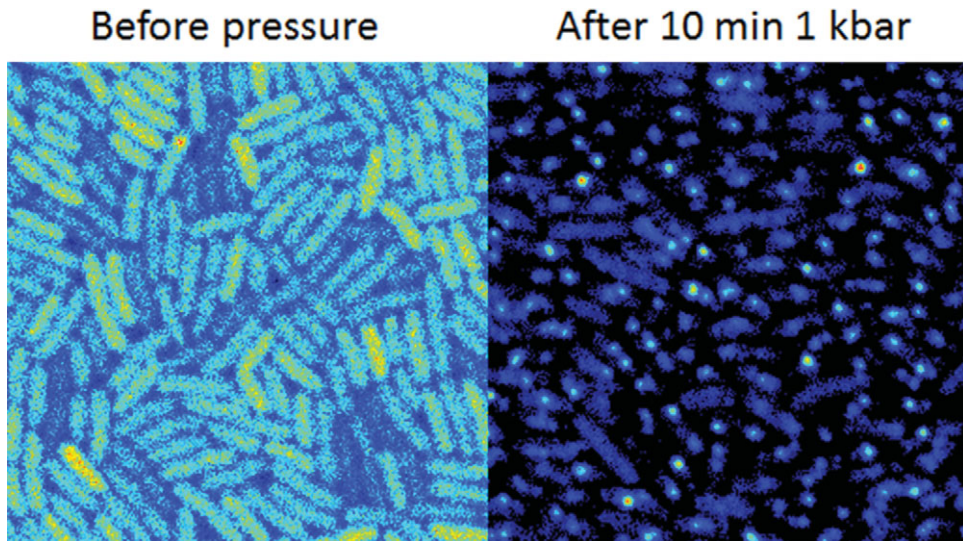

Figure 18.8 Average fluorescence intensity images of GFP-Mrr expressed from an aranbinose-inducible promoter (P_{BAD}) at the natural chromosomal locus in *E. coli* MG1655. (Left) Cells prior to the application of pressure. Full scale is 0–1.48 photon counts per 40 μs. (Right) After 10 minutes at 1 kbar and release of pressure. Full scale is 0.5–6.7 photon counts per 40 μs. Both images are 20 × 20 μm.
Results are similar to those published in (192).

(193,194). Müller and Gratton pioneered the use of capillary tubing for high-pressure microscopy (195) that can withstand pressures up to 6000 bar. However, it has never been applied to live cell imaging. Quantitative analysis of pressure effects on bacterial mobility and flagellar rotation speed were accomplished using a recently designed high-pressure microscopy chamber (196). Unfortunately, owing to the thick windows of this cell, it is not suited for the high-magnification objectives required for advanced quantitative microscopy. This limitation is also the case for two other reported high-pressure microscope chamber designs (197,198). Another hurdle will be the genetic manipulation of extremophile organisms in order to insert fluorescent proteins or other tags into the natural loci of genes of interest.

As in the case of high-pressure molecular biophysics, many unknowns exist regarding the molecular strategies used by cells to adapt to high-pressure or to other extreme conditions. There may be common trends (e.g. dependence on osmolytes) among different types of extremophiles. It is possible that the mechanical properties of high-pressure organisms may be different from those of their mesophilic counterparts or of hyperthermophiles, especially in their membranes and signal transduction pathways. Extreme conditions may affect cell state transitions, growth, differentiation, and development in extremophiles, or even the appearance of multicellularity. Changing geochemical conditions on Earth may have affected cellular phenotypes and the evolution of biological macromolecules.

18.5 Limits to Knowledge and Unknowns

Some of the most fundamental questions about how life on Earth evolved, especially during the first 4 billion years after Earth was formed, relate to how cells and the molecules that keep them alive adapted to changing extreme conditions. Owing to progress in genomics and in molecular and cellular biophysics, the stage is set for a rigorous and systematic examination of some of the most interesting unanswered questions about the evolution of biological systems.

Future work should focus on determining what sorts of physiological properties can be assigned to uncultured subsurface microbes, and perhaps using those insights to develop new techniques to bring them into culture. Linking the experimental work in extreme biophysics with the new frontiers being opened by coupling genomics, transcriptomics, proteomics, and metabolomics with biogeochemical analyses will yield many exiting discoveries about the deep subsurface biosphere in the next few years.

Acknowledgments

Questions for the Classroom

1 What novel techniques have enabled the study of uncultured microbes in the deep subsurface?
2 What are the limitations of some of these novel techniques?
3 What are some general features of metabolism in the deep subsurface biosphere?
4 What are some key ways that high pressure impacts biomolecules?
5 What are some of the novel techniques in use for high-pressure biophysics?

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