Life in deep subsurface

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Abstract. Life extends far deeper into the Earth's subsurface than presumed possible 30 years ago. In the past, it was assumed that life is a surface phenomenon, and that even "hardy prokaryotic types" are not capable of living deeper than tens of meters below the surface [1]. In the 1990s, it became apparent that genetically and metabolically diverse microbial communities existed under highly reducing conditions in the deep subsurface [2]. Today we know that life in the deep subsurface is ubiquitous and comprises a large proportion of the biomass on Earth [3]. Many questions concerning life in the deep remain unanswered. What is the lower depth limit of the deep biosphere? Which energy sources are fueling these communities? How are genetic diversity and functional activity linked to geochemical factors? What we know is that the deep subsurface is an extreme environment and that the microorganisms living here have developed numerous mechanisms to deal with high pressure and temperature, limited energy and nutrient availability, extreme acidity and alkalinity, metal toxicity, and radioactivity [4].

1. Introduction

Prokaryotes are remarkable in their ability to harvest energy from myriad redox reactions [5]. Their metabolic diversity and plasticity allow them to colonize almost all natural and industrial environments. Redox and biochemical reactions catalyzed by bacterial enzymes can lead to mineral precipitation, dissolution or transformation. Therefore, bacteria affect the geochemistry of modern environments, and may have contributed to shaping the near-surface environment of the early earth. Subsurface is classically defined as terrestrial habitats below 8 m and marine sediments below 10 cm. Deeply buried subsurface rocks and sediments may harbour over half of all prokaryotic cells on earth [6]. In this short survey, we will estimate physicochemical constraints affecting microbial life in subsurface, describe tools used to study microorganisms, and then illustrate the previous section by presenting some microorganisms isolated from deep subsurface and some results obtained by molecular studies. We finally discuss about subsurface microbial heterogeneities.

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2. Physicochemical constraints on microbial life in subsurface

2.1 Nutrients

Bacterial energy supply comes from chemical sources, due to fluids that migrate in deep levels in the Earth. This supply lies in the oxidation of an electron donor (H_2, H_2S, CH_4, organic matter…) coupled with the reduction of an electron acceptor (CO_2, SO_4^{2-}, NO_3^-, O_2 when available…) as phototrophy is impossible in subsurface. Microorganisms can be distinguished into physiological groups according to the electron acceptor they use: SO_4^{2-} (sulfate-reducers), NO_3^- (nitrate-reducers), NO_2^- (nitrite-reducers), CO_2 (methanogens), FeIII (Fe-reducers) or O_2 (aerobic to microaerobic). Depending on their carbon source, microorganisms are said heterotrophs when their carbon supply comes from organic compounds, and autotrophs when their carbon supply comes from inorganic carbon. The type of metabolic processes that occurs in subsurface depends to a great extent on the availability of electron acceptors. Considering that stratal waters often contain sulfate at various concentrations, and carbonate, one can assume that the major metabolic processes in oil reservoirs are sulfate reduction, methanogenesis, fermentation, and possibly homoacetogenesis.

Sulfate reduction	$SO_4^{2} + 4\ H_2 + 2\ H^+$	$-> H_2S + 4\ H_2O$
Methanogenesis	$CO_2 + 4\ H_2$	$-> CH_4 + 2\ H_2O$
Fermentation	$C_6H_{12}O_6$	$-> 2\ CO_2 + 2\ CH_3CH_2OH$
Homoacetogenesis	$2\ HCO_3^- + H^+ + 4\ H_2$	$-> CH_3COOH + 4\ H_2O.$

The four corresponding bacterial groups are actually those having been the more frequently isolated from waters sampled from deep subsurface, and are generally considered as potentially indigenous to the subsurface formations. Hydrogen may be supplied for methanogenesis and sulfate-reduction by mineral hydrolysis, maturation of organic matter, metabolic activity of fermenters, or even from the oil aromatization [7]. Water radiolysis was a potential energy source for the deep subsurface biosphere according to [8]. H_2 constitutes a major component of dissolved organic gases (up to 98%) in the groundwater of Precambrian shields, and its concentration ranges up to several mM. These concentrations are several orders of magnitude higher than those observed in shallow aquifers [9]. Radiolysis consists in the decomposition of water due to the products of radioactive decay. Radiolysis has been proposed as a mechanism for generating large quantities of molecular hydrogen, this hypothesis being supported by the fact that H_2-bearing fluid inclusions in quartz are associated with U-bearing [10]. The H_2 yield under conditions that mimic natural radiolysis in subsurface consistent with millimolar H_2 concentrations found in Precambrian shields [8]. As no O_2 or H_2O_2, which are other products of water radiolysis were found in subsurface, they may have abiotically oxidized S^{2-} and Fe^{2+} derived from anaerobic microbial metabolism. This cycling mechanism would maintain the stability of anaerobic conditions as well as supply the electrons acceptors for anaerobic metabolism. The elevated H_2 abundances from radiolysis also enhance abiotic formation of hydrocarbons [11], lipids [12] and organic acids [13], which are thought to be produced by the reactions between CO or CO_2 with H_2 in the presence of metal sulfide catalysts.

Salinity, temperature, pressure and porosity are the main physical constraints on microbial life in subsurface. Bacteria and Archaea can for example inhabit unusual environments with low water availability. Inagaki [14] reported a biogeochemical and microbiological characterization of a microbial community inhabiting deep-sea sediments overlying a natural CO_2 lake, southern Okinawa Trough. They found high abundances ($>10^9\ cm^{-3}$) of

microbial cells (mainly anaerobic methanotrophic archaea and sulfur-metabolizing bacteria) in sediment pavements above the CO_2 lake, decreasing to strikingly low cell numbers (10^7 cm^{-3}) at the liquid CO_2/CO_2-hydrate interface, despite of the potentially aggressive characteristics of this non-polar solvent.

2.2 Salinity

High salt concentrations increase water viscosity and density and decrease gases solubility by an effect called "salting-out". Ions are strongly hydrated, so they tie up a lot of water molecules. That means there is less water free to hydrate the dissolved gas, so less gas can remain dissolved in the salt solution. Microbial life can be found over the whole range of salt concentrations, from freshwater to hypersaline environments (salt lakes, salted food products, chotts, saltern ponds…), where they may reach high population densities [15]. Since biological membranes are permeable to water, cells cannot maintain the water activity of their cytoplasm higher than that of the surrounding environment, which would lead to a rapid loss of water to the environment. To cope with the osmotic stress, microbes developed two different adaptative strategies: the "salt in" strategy, in which cells maintain high intracellular salt concentration (at least equivalent to the external concentration), all intracellular systems being adapted to the presence of salts; the "compatible solute" strategy, in which cells maintain low salt concentrations in their cytoplasm by the action of ionic pumps. The osmotic pressure of the medium is then counterbalanced by organic compatible solutes (glycerol, glycine betaine, trehalose…) [15]. Therefore, life at high salt concentrations is energetically expensive since it involves the buildup and maintenance of steep ion concentration gradients across the cell membrane, whether or not accompanied by the biosynthesis or accumulation of organic osmotic compounds. A survey of the halophilic microorganisms shows that not all known metabolic types function in the presence of high salt concentrations. Denitrification, oxygenic and anoxigenic photosyntheses occur close to NaCl saturation, whereas autotrophic oxidation of NH_4^+ to NO_2^- does not seem to occur above 150 g of salt per liter. Methanogenesis is also sensitive to salt concentration, as reduction of CO_2 with H_2 and acetoclastic split are common in freshwaters and seem not to occur at high salt concentration. Homoacetogenesis and methanogenesis from methylamines seem to be prevalent in hypersaline environments.

2.3 Temperature

Temperature influence on microbial metabolism has been widely studied due to the biotechnological impact of enzymes coming from thermophile microbes. For example, the DNA polymerases of *Thermus aquaticus* or *Pyrococcus furiosus*, two hyperthermophiles, are widely used in PCR (Polymerase Chain Reaction) applications because of their stability when heating the PCR mixture. Microorganisms which inhabit high temperature environments are defined as thermophilic if their optimum growth temperatures are <45°C. If an organism has an optimum and maximum growth temperature of at least 80 and 90°C respectively, it is further defined as a hyperthermophile. Life at high temperature seems to appear in a wide diversity of environment. *Thermocrinis ruber*, an aerobic, facultatively chemolithotrophic bacterium that growths in the laboratory between 44 and 89°C by oxidizing hydrogen, elemental sulfur, thiosulfate, formate of formamide is hosted in an alkaline hot spring in the Yellowstone National Park, USA. Deep-sea hydrothermal systems at a depth of 2600 m on the East Pacific Rise support anaerobic autotrophic methanogens such as *Methanococcus jannaschi*, which grows optimally in the laboratory at 85°C. Acid solutions generated by interactions between volcanic gases and seawater in the Aeolian islands and the solfatara

of Napoli (Italy) are the habitats of acidophilic Archaea, including *Acidianus infernus, Thermoplasma volcanium* and *Metallosphaera sedula*, which grow optimally at pH near 2.

2.4 Pressure

Pressure is a thermodynamic variable that varies from 1 to 1100 atmospheres (0,1 to 102 MPa) from the surface to the deep-sea bottom, and is up to 100 MPa in oil fields. Many thermophiles and hyperthermophiles are also barotolerant/barophiles (or piezophiles in recent terminology) and may employ metabolic processes that are affected by pressure. But at most conditions of biological interest, the effect of pressure on metabolic energetics is secondary to that of temperature [5]. Nevertheless, there is some evidence that elevated pressure may manifest interesting effects on cellular physiology [16]. Hydrostatic pressure in the range of 0.3–0.5 GPa usually inhibits the growth of microorganisms. DNA synthesis is one of the most pressure-sensitive cellular process, especially the initiation of DNA replication [17]. Protein synthesis is also highly susceptible to elevated pressure. Ribosomes associated with both mRNA and tRNA show perfect stability at pressures up to 102 Mpa, although uncharged ribosomes become dissociated at pressures >60 MPa [18]. Therefore, piezophiles are likely to have undergone critical changes in their ribosomal proteins. There is considerable evidence that an increased proportion of unsatured fatty acids in membrane lipids is associated with bacterial growth profiles under high pressure and low temperature [19]. A higher proportion of unsaturated fatty acids would help maintain favourable fluidity and viscosity of biological membranes under high pressures. It is noteworthy that piezophiles present higher metabolic activities under high pressures. Incubating hydrothermal sediments from Guaymas basin under variable pressures, observed higher sulfate reduction rates are higher at 45 MPa than at 1 Mpa in the 75–100°C temperature range [20].

2.5 Porosity

As depth increases, burial of sediments and diagenesis limit space availibility for microorganisms due to the decrease of porosity. So geometrical constraints and mechanical interactions must be considered as well. Small pores restrict bacterial movement and activity, limit nutrient transport and slow the rate of bacterial division. Sediments compaction also leads to spatial isolation due to a lack of pore connectivity, and the decrease of pore-throat size. This implies that all cells in a pore are lineal descendants of a bacterium that became entombed at the time of geologic deposition [21].

3. Tools for analyzing microbial communities

Microbial communities can be analyzed by two major ways, cultivation and culture-independent nucleic acids analyses.

3.1 Culture dependent methods

Bacterial cultures lead to enriched cultures and isolation of pure strains. It is clear that pure cultures do not exist in nature, and that cultivable bacteria only represent a minor fraction of total prokaryotic biomass [22]. In most cases, cultivation-based methods fail to detect the most abundant members of microbial communities in situ. Nevertheless, cultivation of bacterial strains is necessary to access the real physiological mechanisms, and so remain crucial. This is particularly true within the oil field water where there is less biodiversity, as a result of physico-chemical constraints. A most-probable-number

(MPN) method is generally used for the quantitative assessment of microbial communities, with a defined medium containing carbon compounds or not in combination with different electron acceptors targeting different physiological groups (e.g. sulfate-reducers, nitrate-reducers, FeIII-reducers, methanogens and even microaerophiles when present…) or different fermentescible sugars targeting heterotrophic bacteria.

3.2 Culture-independent methods

In parallel, nucleic acids (DNA and RNA) extraction and analyses can be performed. DNAs are large and stable molecules that contain the bacterial genome, thus the potentialities of the microorganisms; RNAs are small and short-lived molecules, formed from DNA, which can be used as a proxy for microorganism activity. Nucleic acids sequences and concentration from deep subsurface can give great informations about microbial ecology and physiology of the microbes (e.g. in situ activities).

A large and diverse suite of protocols has been published on nucleic acids extractions. Two principal approaches exist with their advantages and their drawbacks. The principal approach used consists in an in situ lysis of microbial cells within the environmental matrix (soil, sediment, rock) followed by separation of the nucleic acids from matrix components and cell debris. These methods lead to higher nucleic acids yields and are less time-consuming. But directly-extracted nucleic acids often contain considerable amounts of co-extracted susbtances (such as humic acids in the case of soils) that interfere with subsequent molecular analysis. Furthermore, in certain cases (in particular soils), a proportion of extracted DNA might originate from non-bacterial sources or from free DNA.

Once nucleic acids are extracted and purified, they can be analyzed by way of molecular methods. The principal targeted gene is the 16S rRNA gene. This gene is present in all Prokaryotes. Its product (16S rRNA) is part of the ribosomes which are required by all organisms to synthetize new proteins. It is sufficiently long (1500 bp) to be used as a document for evolutionary history and there is no evidence for horizontal transfer of this gene. As 16S rRNA genes consist of several sequences domains that have evolved at different rates (due to functional constraints), unambiguous alignment of homologous sequences (to define primers and probes) and identification of taxon-specific signatures are possible. However, when considering a single 16S rRNA gene fingerprint band or a 16S rRNA gene partial sequence (see below), the phylogenetic level which this band or sequence represents is not absolutely sure. Many researchers are using the term “Operational Taxonomic Unit” (OTU) to define each band profile or groups of highly related sequences. The use of arbitrarly defined OTU is acceptable from a taxonomic point of view since the definition of bacterial species is itself somewhat arbitrary, as no single marker is also sufficient and necessary [23].

PCR (polymerase chain reaction) is the first step in phylogenetic analysis of microbial communities and for many molecular ecological approaches.

PCR allows the selective amplification of small amounts of DNA extracted from natural samples. The oligonucleotide primers are designed to hybridize regions of DNA flanking the desired gene sequence. Primer selection will depend on the particular application and whether 16S rRNA genes from a wide range of organisms or specific groups are targeted. Repetitive cycles of temperature changes result in an exponential increase in the DNA fragment of interest. Some care must be taken into account when using PCR-based methods. PCR pitfalls are point mutation or deletions, the formation of chimeric PCR products, differential amplification leading to a biased picture of the community [24, 25].

At the end of PCR, the mixture of 16S rRNA genes of comparable lengths can be analyzed several approaches: construction of clone libraries, DNA fingerprinting, and nowadays, high throughput sequencing. DNA sequences can be read and provide information on taxonomic affiliation of microbes as on metabolic capacities.

4. Microbial heterogeneities in subsurface

Improving understanding of the spatial and temporal distribution of microbial organisms and activities is critical due to the difficulty and high cost of obtaining large numbers of well-preserved subsurface samples. The microbiological properties appear to be spatially correlated to geologic, hydrologic and geochemical properties [14, 21]. Subsurface environment often exhibits a pronounced spatial heterogeneity in physical properties as a result of stratified structures and the actions of hydrologic and geochemical processes over geologic time periods. Movements of fluids are strongly controlled by geological setting. As a result, subsurface environments may possess strong contrasts including variations in redox conditions, moisture and nutrients fluxes. Thus, there is potential high heterogeinity in microbiological processes in subsurface. The scale that should be investigated is a function of the scientific question or applied problem under investigation. Studying bioremediation design and engineering, microbial alteration of physical properties and dynamics of microbial colonization should concern strata, laminae and pore/pore network, respectively. Zones of enhanced activity or populations may exist at interfaces of strata where electron donors and acceptors mix [26]. At 90 m depth in a margin site, Pacific Ocean, Prokaryotes had greater activity than in near-surface sediments: this zone corresponded to the sulfate-methane transition, where both sulfate reduction and methane production occurred [26]. The low amounts of methane measured were attributed to anaerobic methane oxidation by a consortium of methane oxidizers (Methanosarcinales) and sulfate reducers (δ-Proteobacteria) [27]. Results indicated that subsurface Prokaryotes were highly active, presented changing diversity associated with interfaces, and that they were active over geological timescales. Geostatistics focuses on the spatial patterns of data, providing tools for characterizing spatial distributions and estimations of variables at unsampled locations. The spatial continuity on which the geostatical approach is based results from the inherent continuity of the processes that control earth sciences phenomena. For example, the geochemical and hydrogeological processes controlling microorganisms distribution in subsurface do not vary randomly, but exhibit strong spatial continuity. Thus, geostatistical models (variogram analysis, cross-correlations...) could increase the ability to model and predict the distributions of microbial properties and the responses of microbial communities to environmental perturbations.

In contrast to studies of surface microbial communities, studies of subsurface environment have not adequately characterized microbial community composition and diversity because of the cost and difficulty of obtaining a large number of samples. It is not clear whether the subsurface microbial communities are closely tied to the surface soil communities, or belong to an independent environment. Subsurface communities are isolated from each other to a greater degree than soil communities. In surface soils, wind transport and flooding can move and mix soils over significant distances, which can not be done in subsurface. Therefore, community differences between sites separated by relatively small distances are potentially greater for subsurface communities. In subsurface, there is the possibility of the existence of some microbial communities linked to the process of sediment deposition, million years ago rather than to their surface counterpart [28].

5. Conclusion

With the development of new high-throughput sequencing technologies, since 2005, pictures of deep subsurface communities from great quality are now being provided, with some obvious results: it seems for example that in certain environments, the communities are dominated by rare taxons [29]. Physiological studies reveal that famine could be the most common lifestyle in deep subsurface [30], communities being prone to reactivation when amended [31].

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