Microbial life in deep subseafloor coal beds

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Deep beneath the seafloor, microbial communities thrive on the leftovers of organic material that in the past settled down from the surface layers of the ocean to the sediment. As the organic matter was buried deeper and deeper over geological time it became increasingly recalcitrant to microbial degradation. Microbial cells that still persist in these ancient deposits appear to survive at the limit of starvation. However, it is estimated that half of all bacteria and most of all archaea in the ocean live under such nutrient-poor conditions in the deep seabed (1). This deep biosphere extends around the globe and, on a long geological time scale, interacts with the chemistry of the sea water. Thus, the nutrients in the ocean and the oxygen in the atmosphere are affected by the efficiency with which subseafloor microorganisms degrade and remineralize the buried organic matter. Microbiologists who study this deep biosphere have been searching for the limits to life in the subsurface and have set new records in finding even deeper and older microbial communities. Trembath-Reichert et al. now report in PNAS (2) that microorganisms live in 20 million-y-old coal beds buried 2 km beneath the seafloor and that the organisms are able to take up nutrients and grow when kept for years under seminatural conditions in the laboratory.

Trembath-Reichert et al. (2) studied deep lignite coal beds that had been discovered by seismic profiling at 1,200-m water depth off the east coast of Japan. A forearc basin is here formed by the subduction of the Pacific Ocean plate, which has gradually also pulled down the continental plate. During the early Miocene, organic remains of rich coastal forests were buried in warm backswamps that later subsided into the cold ocean and formed lignite seams, which were subsequently overlaid by 2-km-thick marine shales. Expedition 337 of the Integrated Ocean Drilling Program (IODP) used the Japanese riser drillship, D/V *Chikyu*, in 2012 to penetrate 2.5 km down into the seabed. They succeeded in recovering sediment core material from the 40–60 °C warm coal beds and shales for microbiological and geochemical studies (3). Among the main objectives was to detect whether deeply buried hydrocarbon reservoirs, such as coalbeds, may act as a geobiological reactor that sustains subsurface life for millions of years. Signatures of life were already indicated by the biogenic isotope signature of methane from that depth and by the relatively low concentrations, relative to methane, of ethane and other hydrocarbons of preferentially thermogenic origin.

The recovery of uncontaminated core material from 2-km depth in the seabed is a truly challenging endeavor. The seabed material used for microbiological studies consisted of crushed pieces of lignite and shale that first needed to be rigorously tested for bacterial contamination from drilling fluid or from handling. This test is critical for the later confidence in the microbiological data. Testing was done by the addition of a perfluorocarbon contamination tracer in the drilling fluid and by checking for seawater- or human-derived bacterial genes (3). The least-contaminated samples were selected, and then even greater methodological challenges started.

The Low Cell Number Problem

The microbial cells in deep marine deposits are small, about 0.5 μm in size (1, 2, 4), and their numbers in the coalbeds were very low: a few tens to thousands per cubic centimeter. A few tens of cells are at the minimum limit of what can possibly be counted under the microscope (5). If we scale this up 100,000-fold for analogy, this cell abundance corresponds to finding and counting a few tens of golf balls in a cubic kilometer of rock. However, by a combination of gradient centrifugation and flow cytometry (6) it was possible to retrieve a large fraction of the cells from the coal samples and extract and sequence their DNA. Interestingly, the bacterial community composition determined from the 16S rRNA phylogenetic marker genes was found to resemble communities from forest soils rather than communities from deep marine sediments (5). This indicates that the indigenous microorganisms in the coalbeds originate from surface communities that

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lived in backswamp forest soils some 20 million y ago. This conclusion was supported by the fact that the DNA was derived from whole cells and not from DNA extracted from bulk sediment, which may also harbor fossil DNA of extinct microbial communities (7).

The metabolic activity of microbial cells in the deep seabed must be exceedingly low in order for organic carbon degradation to persist for millions of years. It therefore remains an often-debated question in deep biosphere research whether most of the observed cells are metabolically active or whether most are in an inactive, dormant state. Mean metabolic rates of subsurface communities have been estimated by modeling or experimentally measuring rates of carbon turnover. The ^{35}S-radiotracer approach to measure microbial sulfate reduction as a terminal organic carbon oxidation pathway enables particularly sensitive experiments. By this method, it was possible to detect the reduction of 1–10 millionths of the sulfate during experiments with deep coalbed samples from IODP Expedition 337 (8). When such rates are compared with numbers of total cells, or of the potentially sulfate-reducing cells, mean cellular rates can be calculated. Under qualified assumptions about the microbial growth yield, assumptions—which admittedly remain poorly constrained—mean generation times in the subsurface have been estimated to range from months to thousands of years (9, 10). Similar rates of microbial biomass turnover have been calculated using a very different approach, based on the spontaneous amino acid racemization, which can be used as a molecular clock (11).

In their new study, Trembath-Reichert et al. (2) have succeeded in performing sensitive laboratory experiments to detect an extremely slow microbial growth in deep coalbed samples. These samples were incubated at 45 °C with stable-isotope–labeled nutrients, which the bacteria could use for metabolism and growth. The nutrients included ^{13}C- and ^{15}N-labeled methanol, methylamine, and ammonium, all of which could expectedly be assimilated by diverse heterotrophic bacteria. Water labeled with the heavy hydrogen isotope, deuterium (^{2}H-H_2O), was also used as a general and very sensitive tracer for growth. The deuterated water is passively incorporated into all cells during their synthesis of new biomass. After 2.5 y of incubation, cells were separated from the coal samples and individual cells were analyzed by nanometer-scale secondary ion mass spectrometry (nanoSIMS) to check whether they had incorporated stable isotope labels. By the very small cell numbers available, such a nanoSIMS analysis is orders-of-magnitude more sensitive than stable isotope probing of bulk-extracted cell constituents. The method requires, however, that the cells can be effectively pulled out of the samples and can be found again for analysis.

The Feeding and Growth of Each Bacterial Cell

A particular advantage of stable-isotope probing using the nanoSIMS is that the isotopic composition of each cell can be determined. The high-resolution nanoSIMS scanning reveals how much organic carbon or ammonium the cells incorporated from the environment into their biomass during the experiment. The incorporation of deuterated water reveals more generally the formation of new biomass, irrespective of the nutrient uptake (12). This isotope information can be used to estimate growth rates, and thus generation times, of those cells that show distinct deuterium enrichment. In the coalbed samples, generation times of such active cells were several months to over 100 y. This is rather similar to the more indirect estimates mentioned above, yet it represents the first direct determinations of deep biosphere generation times by laboratory experiments.

Several important lessons can be learned from the study of Trembath-Reichert et al. (2). First, the subsurface communities may be in a dormant-like state, judging from their extremely low metabolic rates, but they are physiologically intact and on stand-by if nutrients become available. The new data show that a large fraction of the cells were metabolically active or they could be induced to actively assimilate substrate and build new biomass. This active fraction constituted 4–61% of all cells in the 2-km-deep coalbed samples. An earlier study at the same site using similar techniques had shown that the metabolically active fraction at a shallower depth of 200 m in 0.5 million-y-old sediment comprised a much larger fraction, up to 76% of all cells (13).

Second, the cells started to grow slowly, and expectedly to multiply at rates detectable over a few years when the strong carbon and energy limitation of their subsurface environment was experimentally relieved. This does not show to which extent the deep microbial communities also grow and divide in situ, but observations from other deep biosphere studies show that microbially derived amino acids are turning over (11) and that cells are exposed to infection and lysis (death) by viruses (14). Taken together, these data provide strong evidence that they must also multiply to maintain a steady population size.

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Third, a fraction of the community persisted in a truly dormant state in the form of bacterial endospores. This was demonstrated by autoclaving the sediment, a sterilizing heat treatment that generally kills all vegetative cells but may allow some endospores to survive (15). Upon autoclaving and incubating at 45 °C, the persisting community in the coalbed samples was dominated by Firmicutes, a group of bacteria generally able to form endospores. Such endospores of thermophilic bacteria are most probably not a long-term survival form in the subsurface, as they require energy for germination and have been found to slowly decay with a half-life of some hundreds of years (16).

Although the depth and age of the sediment do not appear to set a hard boundary for microbial life in the seabed, high temperature will. The energetic cost of amino acid racemization and the resulting inactivation of proteins have been suggested to restrict microbial life in low-energy environments to an upper temperature limit of approximately 85 °C (17). This limit may, however, have been exceeded during a recent expedition of the D/V *Chikyu* off the east coast of Japan. Elevated cell concentrations were here found at 0.8 km below the seafloor, where the temperature presumably reached 90 °C (18). The extent of microbial life in the seabed thus continues to surprise us and expand the limits of environments known to be inhabited by cells adapted to thrive under extremely low energy flux.

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