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Review

Buried Alive: Microbes from Ancient Halite

Salla T. Jaakkola,[1] Janne J. Ravantti,[1] Hanna M. Oksanen,[1] and Dennis H. Bamford[1,*]

Halite is one of the most extreme environments to support life. From the drought of the Atacama Desert to salt deposits up to Permian in age and 2000 meters in burial depth, live microbes have been found. Because halite is geologically stable and impermeable to ground water, the microbes allegedly have a syn-depositional origin, making them the oldest organisms known to live on Earth. Recently, our understanding of the microbial diversity inside halite has broadened, and the first genome sequences of ancient halite-buried microbes are now available. The secrets behind prolonged survival in salt are also starting to be revealed.

Trends

The first genome sequences of cultivable halite-buried microbes have become available. Most of the genes are highly homologous to those in contemporary halophiles.

Among cultivated halite-buried microbes, archaea belonging to *Halobacterium* and *Halococcus* are common, whereas bacteria are rare. Ribosomal 16S data has revealed many bacteria and unknown archaea in the sediments.

Some halophilic archaea form miniaturized spore-like cells inside halite.

Polyploidy has been observed in halite-buried and contemporary halophilic archaea. Polyploid microbes are efficient in DNA maintenance, which is beneficial for prolonged survival.

Closely related isolates are found in ancient halite and contemporary hypersaline environments. Microbes in halite are likely to contribute to halophilic surface flora, explaining the similarities.

Ancient Life Underground

The biosphere extends far below us. Many underground spaces, for example, the deep sea bottom, ground water reserves, and terrestrial **evaporite basins** (see Glossary) are inhabited by microbes. The total number of underground microbes has been estimated to be around $3.8–6.0 \times 10^{30}$ cells, which is around 90% of all the microbial biomass on Earth [1]. However, little is known about these organisms and their overall impact on our planet's ecology.

Hypersaline environments are rich in halophilic microbes (Box 1). Sometimes the halophiles become encased inside precipitating minerals, such as halite. Viable archaea and bacteria have been isolated from halite that is up to 280 Mya old [2–4]. Interestingly, these cells have been suggested to originate from the same time period as the surrounding minerals. Access to these living fossils and their sequences provides an unparalleled opportunity to study microbial evolution and longevity. In this review, we discuss the recent findings regarding the diversity and survival methods of halite-buried microbes, and the significance of studying microbes inside evaporites.

Why Study Ancient Halite-Buried Microbes?

Halite-buried cells are challenged by starvation, accumulation of end metabolites, deleterious mutations, degradation of cellular components, extremely high ion concentrations, and anoxic conditions. Consequently, they must have qualities enabling their survival within evaporite formations, and novel survival strategies could be discovered. Available sequence data from halite-buried microbes is increasing, opening up new possibilities in halophile research. There are several ribosomal 16S sequences from ancient halite [5–9], and the complete genomic sequence of an Early Cretaceous (123 Mya) *Halobacterium* isolate, *Halobacterium hubeiense*, has been recently published [10]. A draft genome for Permo-Triassic (225–280) Mya *Halococcus salifodinae* (GenBank: AOME00000000.1) is also available. The draft genome of *Halosimplex carlsbadense* (GenBank: AOIU00000000.1) from Permian (250 Mya) rock salt was released in 2013, but the strain originates from an unsterilized sample and might not have an ancient origin [11]. These sequences are opening up a new way to study microbial evolution and longevity. However, more of these ancient genomes need to be sequenced before significant results can be obtained.

[1]Institute of Biotechnology and Department of Biosciences, University of Helsinki, Helsinki, Finland

*Correspondence: dennis.bamford@helsinki.fi (D.H. Bamford).

Box 1. Microbes in Hypersaline Environments

Salt lakes and evaporation ponds with salinity close to the point of precipitation house dense populations of halophilic archaea, bacteria, algae, and their viruses [83]. These waters are usually rich in nutrients and reside in warm climates, supporting the rich microbial flora. Archaea of the family Halobacteriaceae dominate, and species of *Salinibacter* and *Dunaliella* are common among bacteria and algae, respectively. The red and pink coloration associated with saline waters is caused by carotenoid pigments in these cells [5,83]. Halophilic microbe populations are also present in dry terrestrial environments, such as salt deserts and halite deposits [5,17].

Adding to the high ionic strength, many hypersaline environments are affected by strong UV radiation, which can cause mutations. Some salt lakes also experience seasonal drought, and may even dry up completely. Halophilic microbes have many adaptations to overcome these challenges. Light-activated membrane proteins, efficient mutation repair systems, high genomic GC%, acidic proteomes, and elevated intracellular potassium ion concentration are typical features of halophilic cells [84,85].

Evaporite minerals, such as halite, sylvite, and gypsum, form by precipitation in saline waters, and tiny liquid inclusions often form inside them. Inclusions in halite are typically of micrometer scale, and their abundance may reach up to 10^{10} inclusions per cm^3 [30]. Sometimes microbes become trapped inside these inclusions, where liquid water allows them to remain viable and wait for the environmental conditions to become more hospitable [56]. The topmost mineral layers at the anoxic bottom of salt lakes are commonly colonized by anaerobic archaea and bacteria, which are referred to as endoevaporitic communities [86].

Since halite is chemically stable and has a low water permeability and heat conductance, it is used for storing radioactive waste. However, the heat-emitting waste may affect surrounding minerals and biological material and liquid pockets inside them. The risks related to microbial activity have also been considered, especially concerning the microbe-mediated motility of radionuclides [12,13]. The Waste Isolation Pilot Plant in the Salado formation in New Mexico has been shown to house microbes with high phenotypic diversity [14]. The facility is designed to hold various types of waste, including cellulose, which is consumed by various microbes in the sediments [14]. Because metabolically active microbes could cause corrosion of waste containers or build up pressure with gases, it is important to study the risks related to the microbial communities inhabiting the repository sites so that the biological aspect can be taken into account when designing safe solutions for long-term waste-deposition. There is also a possibility of life existing in the subsurface of other planets and celestial bodies [15]. For example, the now-frozen water on Mars may have been liquid in the past, offering a prerequisite for life. There is evidence of evaporitic minerals on the planet [16], and if there ever was life on Mars, remnants of it could now be in slumber under the surface. Spectroscopic techniques have been tested for searching for signs of life in dry halite environments [17], and are intended to be used on Mars as well. Other potential targets for searching for halophilic life in the Solar System are Jupiter's moon Europa and Saturn's moon Enceladus [18,19]. Identifying biological signatures of halophiles in extraterrestrial halite would support the theory of panspermia and an extraterrestrial origin of life [17]. All in all, ancient halite is a fairly unknown habitat, and the overall significance of buried microbes is still uncertain. They might reveal unknown stories from the history of life, perhaps even on other planets, and might help us to understand mechanisms of defying time.

The Age and Origin of Microbes in Halite Sediments

Halite deposits are found all across the world. Microbial isolates and ribosomal 16S sequences have been retrieved from halite sediments in North and South America, Europe, and Eastern Asia (Figure 1 and Table 1). There are two main methods used for halite sampling. First, crystals can be cut or blasted off from freshly exposed mine walls; this is easy and inexpensive. However, these sites have already experienced disturbances. For example, the change in pressure near mine shafts and tunnels may affect fluid movement in halite. Second, drill-core sampling allows us to obtain material from great depths at undisturbed sites, but the costs are high. The drilling fluid might also introduce contaminants into the bore hole, and the outermost layers of the drill cores may become colonized by outside microbes.

To ensure that the isolated cells and extracted DNA originate from ancient material, the halite crystal surfaces need to be sterilized. Methods using ethanol and/or a Bunsen lamp have been

Glossary

Biomass: the total mass of all living organisms.
Cosmogenic isotope: rare isotopes, such as carbon-14, beryllium-10, and chlorine-36, created by high-energy cosmic rays. These isotopes are produced at a known rate, and can be used for radiological dating.
Endolithic/-evaporitic microbes: microbial cells living inside rocks or evaporitic minerals, such as halite or gypsum.
Evaporite basin: a geological formation of buried minerals that have formed by precipitation.
Halite: a mineral form of sodium chloride, also called rock salt.
kya: thousand years ago, or thousand years of age.
Mya: million years ago, or million years of age.
Necromass: biomass of dead organisms.
Syndepositional or synsedimentary: material that has been deposited simultaneously with sediment formation.