



## Microbial life of the deep saline biosphere

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### ABSTRACT

Transfer microbiological studies under the surface of the Earth to exploration of deep intraterrestrial microbial life is important to allow exceeding the current framework of science. Microbial life is determined by physical, geochemical, and biological factors such as the availability of liquid water, energy, nutrients, and trace elements. Additionally, conditions including ambient temperature, pH, salinity, and pressure interact with biological systems to define the space for life. Recent studies have shown the presence and activity of cells in deep marine sediments and in the continental deep biosphere. Characterizing the bacterial community composition and recognition of diversity of microorganisms may play an important role in biogeochemical carbon cycling and potential biotechnological application of these microorganisms.

### Indexing terms/Keywords

salinity environment, microorganisms, halophile, methanotrophs

### DEEP SALINE BIOSPHERE

Under the earth, there are many factors limiting microbial life such as low oxygen levels, water and nutrient availability. With an increase in depth, photosynthetically derived organic carbon becomes limited and hardly accessible [49]. Extreme conditions are also associated with pressure, temperature, pH, salinity. Despite this, the deep subsurface microbial communities are very diverse, spanning all domains of life [3, 9, 10, 41]. When talking about deep saline biosphere we mean mainly salt mines, where it is or was carried on exploitation of salt deposits. Therefore, access to the research materials is easier. All over the globe, numerous underground salt mines are in operation. Underground rock salt mining has developed on the greatest scale in the USA and Canada. The most data on underground salt mines come from Europe (Austria, France, United Kingdom, Ukraine, Poland, Spain, Italy, Romania). Every mine is a potential habitat for valuable extremophiles. In recent times, development of molecular techniques has allowed identification of microorganisms existing in particularly adverse conditions and explanation of the mystery of life in these conditions. In our opinion particularly interesting may be the salt mine in Wieliczka (Poland), which was included on the First International List of the World Cultural and Natural Heritage UNESCO. This salt deposits have been exploited for over 750 years in underground mines. After 1996 exploitation of salt has finished. Microbial research related to the Wieliczka Salt Mine is unique, since to our knowledge, only a few researchers have investigated the animated world of Wieliczka. The first main recognition of microorganisms in brines from Wieliczka Salt Mine was done in 1913 [42]. However, the research was limited only to brine investigations. Due to the limited access to the publications, the results of the microbial identification are still not known. Only Radax et al. reported on strain PW5. 4, originating from Wieliczka Salt Mine water [52]. Later studies about the microfauna from Wieliczka Salt Mine were concentrated on assemblages of foraminifers [40] and were related to recognition of novel species of amoeba - *Selenaion koniopes* [47]. Recently, two halophilic *Archaea* strains of *Halorhabdus rudnickae* sp. nov., were isolated from a sample taken from a borehole in the Barycz mining area belonging to the Wieliczka Salt Mine Company [2].

### MICROBIAL LIFE IN SALT MINE

In the world literature, it is possible to find information about the identification of microorganisms inhabiting different salt mines. In 1960, Reiser and Tasch isolated bacteria with brine (Permian salt mine, Kansas) [53], then Nehr Korn and Schwartz identified microorganisms from Braunschweig mine [43]. Furthermore, Vreeland and Huval, who investigated brines formed by dissolution of salt deposits by surface water, isolated many strains of haloversatile types (0±17% (w/v) NaCl), moderate halophiles (2±20% (w/v) NaCl), and extreme halophiles (12±32% (w/v) NaCl) [59]. In Winsford salt mine (UK), the research was conducted [44, 45, 54]. Various kinds of moderately/extremely halophilic bacteria and Archaea have been isolated from ancient salt deposits, in which several new taxa were identified [44, 45]. Dombrowski and Tasch were the first who isolated microorganisms from rock salt [20, 59]. Reiser and Tasch succeeded in isolating diplococci, which they had observed in fluid inclusions in Permian salt (Kansas, USA) [53]. Dombrowski performed similar work on Permian rocks in Europe and older rocks worldwide [20]. Enrichments in salt-saturated medium yielded a strain from several sites resembling *Bacillus circulans* [20]. Another isolate called *Pseudomonas halocrenaea* was subsequently shown to be indistinguishable from *Pseudomonas aeruginosa* [19] and must therefore be considered as a contaminant. Although they were able to see fossil bacteria, Schwartz and colleagues failed to isolate microorganisms from 30 samples of rock salt [7]. Bibo and coworkers attempted to repeat Dombrowski's isolations with numerous controls and tests of surface sterilization. They isolated extreme halophiles described as Gram-positive cocci and rod-shaped spore formers from primary Permian Zechstein salt cores [6]. Norton et al. isolated seven haloarchaeal strains from rock



salt in Winsford salt mine; three belonged to the genus *Halorubrum* and four to *Haloarcula*. The next microorganisms were isolated in the Permian Salado formation, New Mexico [60], Permian Zechstein deposit mined at Bad Ischl, and preliminary characterization places them in the genera *Halococcus*, *Haloarcula* and *Halorubrum* [56]. The latter two genera were also found in rock salt from Winsford [45]. In a recent study of the microbial diversity of the salt deposit of the Yipinglang salt mine in south-west China, a moderately halophilic, facultatively alkaliphilic strain YIM Y15T *Salinicoccus kunmingensis* sp. nov. was isolated from a brine sample [16]. Next, Yang et al. isolated *Thermobifida halotolerans* sp. nov. in the sample from the same mine [63]. Akhtar et al. conducted research on biodiversity and phylogenetic analysis of culturable bacteria indigenous to Khewra salt mine of Pakistan. They found species similarities with species of the genus *Bacillus*, *Escherichia coli*, *Staphylococcus arlettae*, and *Staphylococcus gallinarum* [1]. Moreover Xiao et al. isolated *Roseivivax sediminis* sp. nov., a moderately halophilic bacterium from the sediment from a salt mine in China [62]. At the same time, Fodinibus *salinus* gen. nov., sp. nov., another moderately halophilic bacterium was isolated in China by Wang et al. [61]. Chauhan and Garlapati reported on bacterial strain *Staphylococcus arlettae* JPBW-1 isolated from rock salt mine in Darang, India. This strain produces lipase, which may have considerable potential for industrial application from the perspectives of its tolerance towards industrial extreme conditions of pH, temperature, salt, and solvent [11]. In 2012, Yildiz et al. isolated and characterized 8 different isolates from a salt mine located in central Anatolia, Turkey [64]. These are only a few examples of research on the identification of microorganisms from salt mines. Recent studies shows that microbial communities in deep subsurface environments are very popular because of many unexplained questions related to the mystery of deep biosphere life. Miettinen and coworkers described microbial and geochemical processes in a deep subsurface in Pyhäsalmi mine, Finland. Bacterial, archaeal, and fungal diversity in deep groundwaters, where total dissolved solids (TDS) range from 17.7 to 76.2 gL<sup>-1</sup> was confirmed [41]. In 2016 year scientists isolated a novel extremely halophilic archaeons belonging to the genus: *Halorubrum*, *Halobaculum* and *Haloparvum* from a salt mine in Yunnan, China [12-15].

## THE MECHANISM OF ADAPTATION TO THE SALINITY ENVIRONMENT

The mechanism that microorganisms have evolved to survive in saline environments accompanied by restricted water supplies is still a problem studied by many researchers. Cells exposed to osmotic stress, such as an increase in osmotic pressure, initiate mechanisms of active coping with the adverse consequences of its effects. Osmoregulatory processes are designed to maintain cell turgor, hence ensuring proper conditions for bacterial growth. Osmoregulation, which consists of maintenance of fluid and electrolyte balance of cells, requires accumulation of specific compatible solutes (osmolytes) and inorganic ions, mainly K<sup>+</sup> and Cl<sup>-</sup>. The concentration of osmolytes in a bacterial cell can vary from millimoles to 1-2 M. Many different small molecules are known as organic osmolytes and other compatible solutes. These solutes fall into a few major chemical categories: small carbohydrates including sugars (e. g. trehalose), polyols (glycerol, inositols, sorbitol, etc.), amino acids (glycine, proline, betaine, glutamate, etc.), and derivatives (e. g. ectoine, hydroxyectoine); methylamines (such as N-trimethylamine oxide), glutamate [23, 48, 50]. The type of osmolytes and the mechanism of uptake thereof from the environment as well as their impact on the growth and development of cells are different depending on the type of microorganism. Gram-positive and Gram-negative bacteria differ in preference for the type of osmolytes. Under osmotic stress, Gram-negative bacteria store mainly glutamate and K<sup>+</sup>, while Gram-positive bacteria accumulate amino acids, mainly proline. In some halotolerant, halophilic, and methylotrophic microorganisms, including methanotrophs and other bacteria of the genus: *Brevibacterium*, *Streptomyces*, *Bacillus*, *Marinococcus*, *Sporosarcina*, *Chromohalobacter*, *Halomonas*, and *Vibrio* accumulation of ectoine is mainly observed [4, 17, 35].

## THE IMPORTANCE OF HALOPHILIC AND HALOTOLERANT MICROORGANISMS

Numerous studies have confirmed that halophilic microorganisms are capable of producing β-carotene from *Dunaliella*, bacteriorhodopsin from *Halobacterium*, and ectoine from *Halomonas* [46]. Recently, many novel and unique molecules (e. g. enzymes, bacteriorhodopsins, etc.) have been explored in these organisms, which are usable for molecular biotechnological applications [18, 46]. A most useful halophilic biomolecule is the retinal-containing chromoprotein, being used for diverse applications, such as in biocomputing [8], as a light sensitive neurological probe and for treatment of blindness from retinitis pigmentosa [24]. Halophilic microorganisms also produce many stable enzymes (including hydrolytic enzymes such as DNases, lipases, amylases, gelatinases, and proteases) capable of functioning under conditions that lead to precipitation or denaturation of most other proteins. Enzymes are used in industry for the production of pharmaceuticals, foods, drinks and confectionery, as well as in textile and leather treatment, paper industry, and wastewater treatment. Compatible solutes of halophilic bacteria used in cosmetics to improve hydration properties [5] are also important. Moreover, halophilic proteins compete effectively with salts for hydration, a property that may result in their functioning in low water activity environments, including organic solvents. Biodegradable polymers, such as polyhydroxyalkanoates are produced in large quantities by some halophilic microbes [25], and have both medical and other specialized applications. A variety of other novel halophilic biomolecules have been targeted for commercial applications, e. g. gas vesicles for bioengineering floating particles, pigments for food colouring and metabolites as stress protectants.

## METHANOTROPHIC BACTERIA

One of the groups of microorganisms inhabiting deep saline biosphere can be methanotrophic bacteria. To our knowledge, methanotrophs that can colonize such an ecological niche have not been isolated yet. Methanotrophic bacteria are a subset of a physiological group of bacteria known as methylotrophs [26]. Phylogenetically, methanotrophs belong to α- or γ- Proteobacteria, but there are also some representatives affiliated as Verrucomicrobiales. These organisms are unique in their ability to utilize methane as a sole carbon and energy source and play an important role in the global biogeochemical cycles of carbon and nitrogen. A majority of known methanotrophic species are nonhalophilic neutrophiles [26]. Halotolerant and halophilic methanotrophs have been isolated from seawater, coastal lagoons, and



several soda lakes [38, 37, 33]. Among salt-depend methanotrophs, *Methylobacterium alcaliphilum*, *M. buryatense*, *M. kenyense*, *Methylobacter marinus*, *M. modestohalophilus* have been isolated [30, 31, 32, 34, 58]. Moreover, Doronina et al. isolated methanol and methylamine-utilizing bacteria from the genera *Methylophaga* and *Methylarcula* [21, 22]. Only a few strains of marine methanotrophs growing at salinities of 0.15–4% NaCl have been isolated [38, 37]. Furthermore, Heyer and colleagues isolated two strains of methanotrophic bacteria from hypersaline lakes in the Crimean Peninsula of Ukraine. Strains 10KiT and 4Kr are moderate halophiles that grow optimally at 5. 8–8.7% NaCl and tolerate a NaCl concentration from 1.2 to 15 % [27].

Methanotrophs have a great potential for use in industry, biotechnology and bioremediation. This may relate to production of osmoprotectants - ectoine. The natural source of ectoine may be irreplaceable in a variety of medical and cosmetic preparations. Methanotrophic bacteria are also able to synthesize other substances e. g. methanol [36], PHB [65], and cobalamin [28]. An enzyme found in methanotrophs responsible for oxidation of CH<sub>4</sub> is methane monooxygenase (MMO), which catalyses the conversion of methane to methanol [26, 39], and can participate in the biotransformation and bioremediation of substances such as alkanes, alkenes, aliphatic and aromatic substances, or their derivatives such as TCE (trichlorethylene) [51].

Due to promising applications of extremophiles further research and exploration of new strains are necessary [29]. Generally, subsurface environments should receive much more attention because their microbial inhabitants may play an important role in biogeochemical carbon cycling. These findings hold large implications for understanding the ultimate limits for life on Earth and the potential for life to exist in the subsurface of other planets, such as Mars [55].

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