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## History of discovery of the first hyperthermophiles

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**Abstract** Hyperthermophiles, growing optimally at 80°C and above had been discovered in 1981. They represent the upper temperature border of life and are found within high temperature environments. In their basically anaerobic surroundings, they gain energy mainly by inorganic redox reactions. Within the phylogenetic tree, hyperthermophiles occupy all the short deep branches closest to the root. The earliest archaeal phylogenetic lineage is represented by the extremely tiny members of the novel kingdom of Nanoarchaeota.

**Keywords** Hyperthermophiles · Archaea · Phylogeny · Cultivation

### Introduction

Since Louis Pasteur it had been generally accepted that vegetative cells of bacteria are safely killed at temperatures between 80 and 100°C. The Pasteurization technology is based on this observation. In 1980, when Wolfram Zillig and I became interested, thermophiles had been known already since a long time. A few of which grow optimally (fastest) at temperatures up to 75°C (Brock 1978). In following Pasteur's principle, similar to mesophiles they are all killed by incubation at 100°C. At that time, the organism with the highest growth temperature had been *Sulfolobus acidocaldarius* (Brock et al. 1972). It thrives aerobically at 75°C within acidic hot mud ponds in Yellowstone National Park and exhibits an upper temperature limit of growth at 85°C. In addition, Tom Brock had already reported on non-culturable rod-shaped microbes [now: *Thermocrinis* (Huber et al. 1998)] growing in boiling hot springs with neutral pH in Yellowstone National Park (92°C, due to

the high altitude) (Brock 1978). *Sulfolobus* had been commonly seen as a highly derived species, a kind of curiosity (mis-classified) among the Pseudomonads. Its aerobic lifestyle with its suggested much higher yield of energy appeared essential to resist thermal destruction (Castenholz 1979). The observed much lower growth temperatures within the anaerobic thermophilic methanogens seemed to confirm this prejudice. In sea water, the high salt concentration was taken as additional stress preventing an extremely hot lifestyle (Castenholz 1979). Therefore, the possibility of anaerobic extreme thermophiles within boiling terrestrial and marine environments had never been taken into consideration. At that time, Carl Woese had just published his revolutionary concept of a three domain living world and his discovery of the Archaea (Woese and Fox 1977). As Woese had found out, *Sulfolobus* in reality belonged to the Archaea domain. Within there, it represented the most deeply branching-off lineage. The RNA polymerase work together with Wolfram Zillig confirmed Woese's finding (Zillig et al. 1979). Therefore, this novel view on universal phylogenetic relationships had convinced me rather early and became essential for my thinking.

Here, I will introduce the discovery and properties of hyperthermophiles (Stetter 1992) which we were able to isolate during the last 25 years. They grow fastest between 80 and 106°C and are mainly strictly anaerobic Archaea. Some hyperthermophiles grow even at 113°C and survive autoclaving (Blöchl et al. 1997).

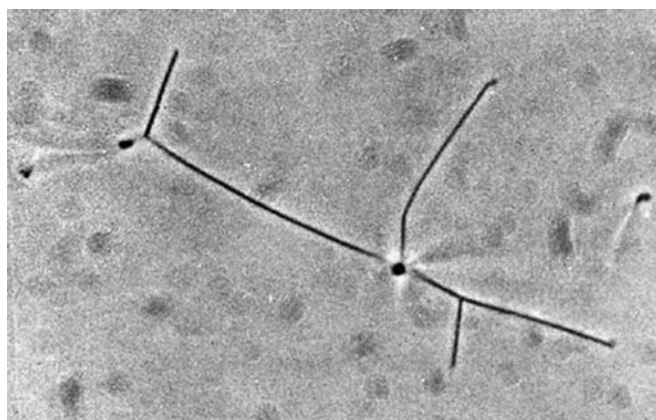
### Discovery

On our first trip to Iceland in 1980, by field microscopy Wolfram Zillig and I inspected boiling (98–100°C) springs and mud pools in several areas. Surprisingly, a great deal were teeming of microorganisms with very unusual shapes like antler-shaped cells with true branchings [Fig. 1; later: *Thermoproteales*; (Zillig et al. 1981)]. When I poured the redox indicator resazurin into such boiling environments, the blue clouds became

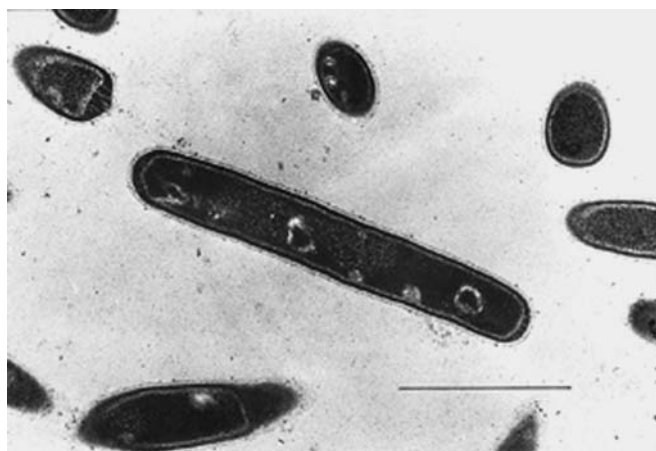
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immediately reduced, indicating that they were highly anaerobic. I took several samples of boiling water and mud. In order to keep them anaerobic, after adding resazurin, sodium sulphide and sodium dithionite, I enclosed them in storage bottles with tightly fitting stoppers. From a sample taken from a strongly gassed little waterhole in the Kerlingarfjöll mountains, I was able to isolate *Methanothermus fervidus*, a novel rod-shaped methanogen [Fig. 2; (Stetter et al. 1981)]. For the first time, this organism grew at a temperature of up to 97°C and exhibited its fastest (optimal) growth at 82°C. Therefore, surprisingly this strictly anaerobic archaeon grew at much higher temperatures than the aerobic *Sulfolobus acidocaldarius*. It became the key organism of my thinking outside the box. From the anaerobic samples taken at this trip, Wolfram Zillig and I were able to isolate the first members of the strictly anaerobic *Thermoproteales* (Zillig et al. 1981). Similar to *Methanothermus*, the *Thermoproteales* exhibited growth temperature of up to 97°C and were unable to grow at 65°C or below. Therefore, the novel isolates exhibited a so far



**Fig. 1** Antler-shaped cell of *Thermoproteus* with true branchings, about 100 µm in length and 0.4 µm in diameter. Phase contrast light micrograph



**Fig. 2** *Methanothermus fervidus*, ultrathin section. Transmission electron micrograph. Scale bar, 1 µm

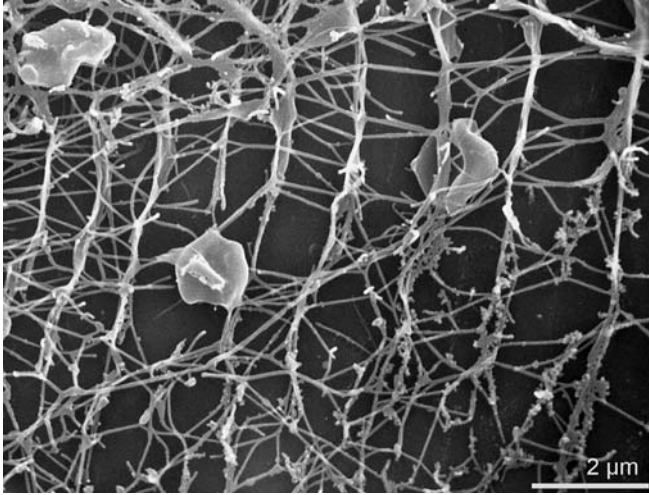
unknown order of magnitude of extreme thermophily. In order to verify my assumption of life possibly existing at temperature above 100°C, during my holidays in 1981 at Vulcano Island (Italy), I took samples from a submarine solfataric field situated at the hot sea floor at Porto di Levante (Fig. 3). As a result of the high hydrostatic pressure, water remained liquid at a temperature higher than 100°C. From these samples, in the laboratory members of the novel strictly anaerobic *Pyrodictium* grew up (Fig. 4). For the first time, these organisms were able to grow above 100°C in superheated water with an optimal growth temperature of 105°C and an upper limit at 110°C (Stetter 1982). Based on their Vulcano-adapted primitive lifestyle, I raised the hypothesis that similar hyperthermophilic organisms could have existed already at the early Earth, 3.9 billion years ago. At those Hadean times, due to a still very brittle crust and very active volcanism, Earth had been much hotter than today. Based on my new cultivation experience, in order to find more of those exciting hyperthermophiles, during the last 25 years I visited high temperature areas all over the world and isolated high temperature organisms from there. At present, hot environments like terrestrial and submarine heated soils, sediments and hot springs are mainly found in areas of active volcanism along tectonic fracture zones and hot spots. I had visited several of these sites including deep sea hot vents with their spectacular “black smokers”. In addition, I discovered communities of hyperthermophiles within deep subterranean (non-volcanic) geothermally heated oil-bearing sandstone and limestone with in situ temperatures of about 100°C some 3,500 m below the bottom of the North sea and the surface of the Alaskan North slope permafrost soil (Stetter et al. 1993). So far, my lab isolated and described about 50 new species of hyperthermophiles, among those representatives of the novel bacterial genera *Thermotoga*, *Thermosipho*, *Aquifex*, *Thermocrinis* and archaeal genera



**Fig. 3** Sampling at the submarine hydrothermal vents at Porto di Levante, Vulcano, Italy. Gasses and hot water escape from cracks within hot liparitic rocks

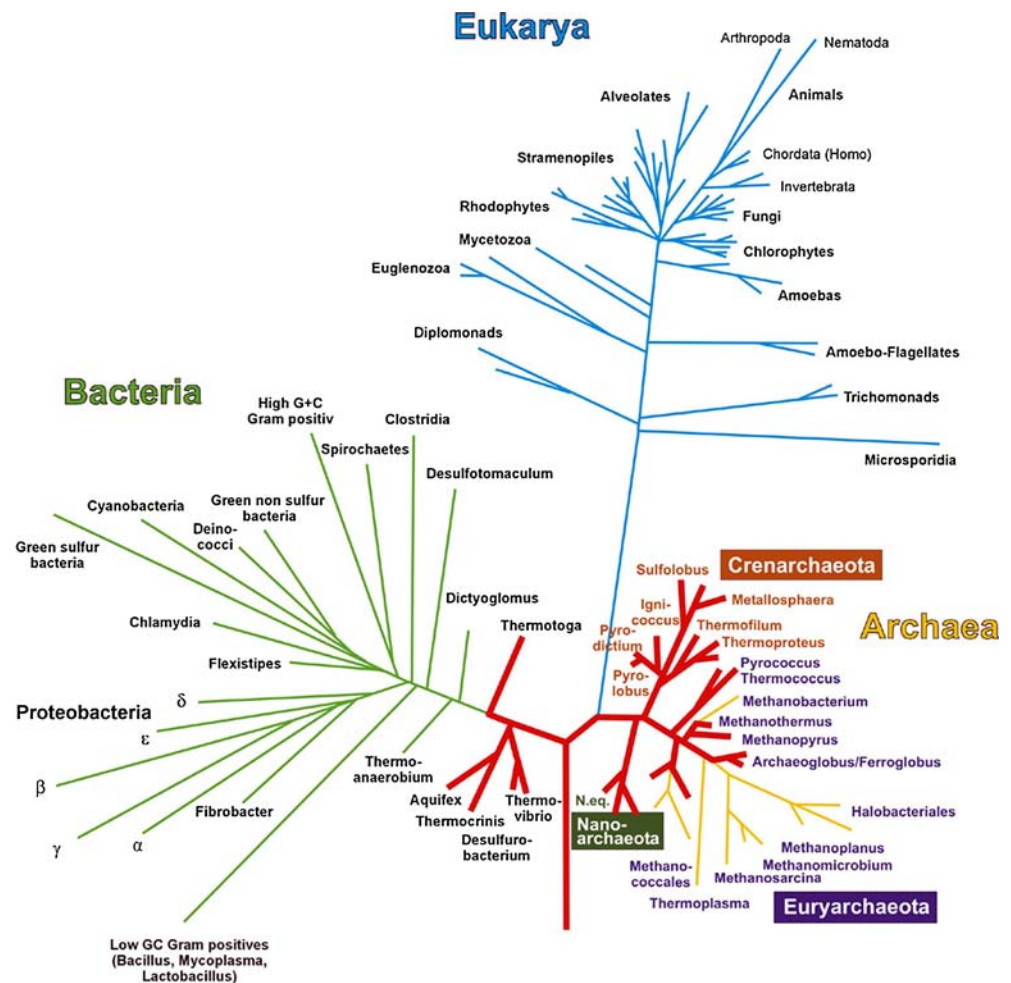
*Acidianus*, *Metallosphaera*, *Stygiolobus*, *Thermoproteus*, *Pyrobaculum*, *Thermofilum*, *Desulfurococcus*, *Staphylothermus*, *Thermosphaera*, *Ignicoccus*, *Thermoplasma*, *Pyrodictium*, *Pyrolobus*, *Thermococcus*, *Pyrococcus*, *Ar-*

*chaeglobus*, *Ferroglobus*, *Methanothermus*, *Methanopyrus* and *Nanoarchaeum* [overview: (Stetter 2005)]. In Woese's small subunit rRNA-based phylogenetic tree (Fig. 5), all extremely short and deeply branching-off lineages within the archaea and bacteria are exclusively represented by hyperthermophiles, indicating a slow rate of evolution.



**Fig. 4** *Pyrodictium abyssi*, disk-shaped cells within a network of ultrathin tubules. Scanning electron micrograph

**Fig. 5** Small subunit rRNA-based universal phylogenetic tree. The red bulky lineages represent hyperthermophiles





Enrichment cultures can be obtained by simulating the varying geochemical and geophysical conditions of the environment. Various plausible electron donors and acceptors may be used under anaerobic, microaerophilic, or (rarely) aerobic culture conditions. Depending on the (unknown) initial cell concentration and the doubling time of the organism, positive enrichment cultures of hyperthermophiles can be identified by microscopy within 1–7 days. For a deeper understanding of the organisms, the study of pure cultures is required. Due to the high incubation temperatures, the traditional way of cloning by plating does not perform well. Therefore, we developed a new procedure in order to clone single cells anaerobically under the laser microscope by employing optical tweezers (Ashkin and Dziedzic 1987; Huber et al. 1995). Large cell masses are required for biochemical and biophysical investigations. For mass culturing of hyperthermophiles, in collaboration with an engineering company, a new type of high temperature fermentor was developed (Fig. 6). Its steel casing is enamel-protected in order to resist the highly corrosive culture conditions. Sharp-edged parts like stirrers, gassing and sampling pipes and condensers are made of titanium. The cell yield of a 300 l fermentation may vary from about 3 g to 2 kg (wet weight), depending on the hyperthermophilic isolate.

### Energy sources and lifestyle

The energy sources of hyperthermophiles are very simple. Most species exhibit a chemolithoautotrophic mode of nutrition (Fig. 7). Anaerobic and aerobic types of respiration follow inorganic redox reactions (chemolithotrophic), and  $\text{CO}_2$  is the only carbon source required to build up organic cell material (autotrophic). Molecular hydrogen serves as an important electron donor. Other electron donors are sulphide, sulphur, and

ferrous iron. In some hyperthermophiles oxygen may serve as an electron acceptor. However, these organisms are usually microaerophilic. Anaerobic respiration types are the nitrate-, sulphate-, sulphur- and carbon dioxide-respirations. While chemolithoautotrophic hyperthermophiles produce organic matter, there are some obligate heterotrophic hyperthermophiles which depend on organic material as energy- and carbon-sources. In addition, several chemolithoautotrophic hyperthermophiles are opportunistic heterotrophs. Heterotrophic hyperthermophiles gain energy either by aerobic or different types of anaerobic respiration using organic material as electron donors, or by fermentation.

Hyperthermophiles are adapted to distinct environmental factors including composition of minerals and gasses, pH, redox potential, salinity and temperature. They grow-similar to mesophiles-within a temperature range of about 25–30°C between the minimal and maximal temperature. Fastest growth is obtained at their optimal growth temperature which may be up to 106°C. As a rule, hyperthermophiles do not propagate at 50°C or below, some not even below 80 or 90°C. Although unable to grow at ambient temperatures, they are able to survive there for many years. Based on their simple growth requirements, hyperthermophiles could grow on any hot water-containing site, even on other planets and moons like Mars and Europa.

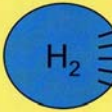
### Examples of recent hyperthermophilic isolates

Within the Bacteria domain, the deepest phylogenetic branch is represented by the hyperthermophilic *Aquifex* (Stetter 1992). Its type species *Aquifex pyrophilus* (Huber et al. 1992) is a motile rod-shaped chemolithoautotroph (Fig. 8). It is a facultative microaerophilic anaerobe: under anaerobic conditions, *Aquifex pyrophilus* grows by nitrate reduction with  $\text{H}_2$  and  $\text{S}^0$  as electron donors.



Fig. 6 Hyperthermophiles fermentation plant, University of Regensburg. Partial view, showing two 300 l and one 130 l fermentors

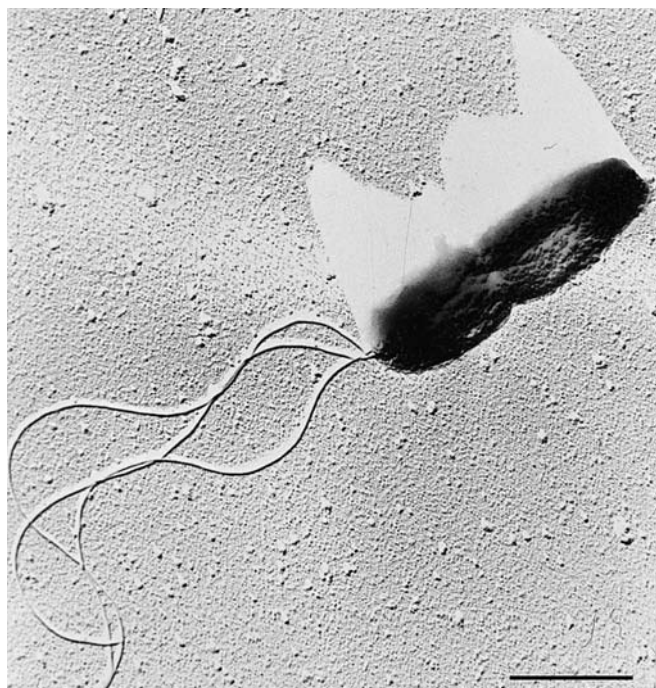
### Energy-yielding reactions of Hyperthermophiles

Electron Donor	Electron Acceptor	Product
 $\text{H}_2$	• $\text{CO}_2$	Methane
	• $\text{Fe}(\text{OH})_3$	Magnetite
	• $\text{S}^0; \text{SO}_4^{2-}$	Hydrogen Sulfide
	• $\text{NO}_3^-$	Nitrogen ( $\text{NH}_3$ )
	• $\text{O}_2$ (traces)	Water
$\text{S}^0$ (Pyrite)	• $\text{O}_2$	$\text{H}_2\text{SO}_4$ (+ $\text{FeSO}_4$ )

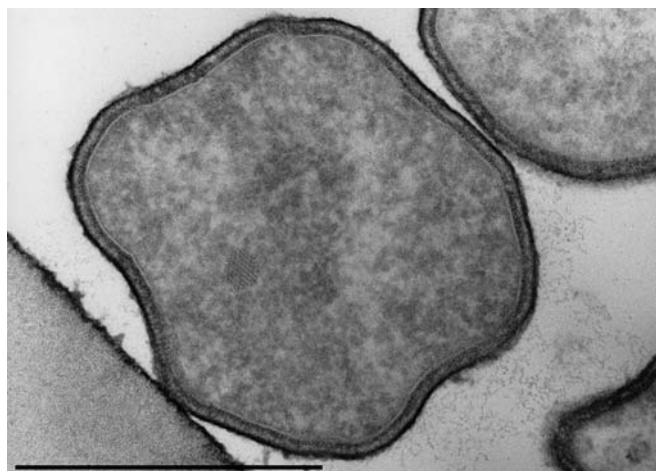
Source of Cell Carbon:  $\text{CO}_2$

Additional Growth requirements:  
Heat, Trace minerals, Liquid water

Fig. 7 Main energy sources in chemolithoautotrophic hyperthermophiles

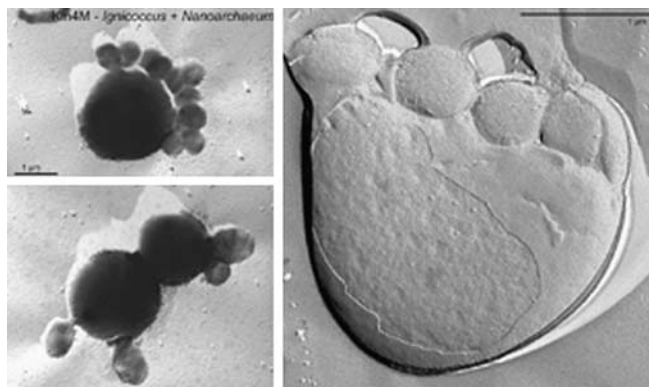


**Fig. 8** *Aquifex pyrophilus*. Dividing flagellated cell. Pt-shadowing. Transmission electron micrograph. Scale bar, 1  $\mu$ m



**Fig. 9** *Pyrolobus fumarii*. Lobed coccoid cell. Ultrathin section. Scale bar, 0.5  $\mu$ m

Alternatively, at very low oxygen concentrations (up to 0.5%, after adaptation) it is able to gain energy by oxidation of  $H_2$  and  $S^0$ , using oxygen as electron acceptor. Members of *Aquifex* are found in shallow submarine vents. *Aquifex pyrophilus* grows up to 95°C, the highest growth temperature observed within the bacteria. From the walls of a black smoker at the Mid Atlantic Ridge, we had isolated the Archaeon *Pyrolobus fumarii* (Blöchl et al. 1997). Cells are lobed cocci about 0.7–2.5  $\mu$ m in diameter (Fig. 9). *Pyrolobus fumarii* is optimally adapted to temperatures of superheated water, exhibiting an optimal growth temperature of 106°C and



**Fig. 10** *Nanoarchaeum equitans* (tiny cocci) attached to *Ignicoccus hospitalis* (large cocci). Left: Pt-shadowing. Right: Freeze-etching. Transmission electron micrographs

an upper temperature border of growth at 113°C. It is so dependent to high temperatures that it is unable to grow below 90°C. Cultures of *Pyrolobus fumarii*, similar to *Pyrodicticum occultum* are able to survive autoclaving for one hour at 121°C. Finally, I want to introduce the pheno- and genotype of a member of a novel group of hyperthermophilic virus-sized Archaea which may have already existed at the dawn of life and which had been completely overlooked, so far. From a submarine hydrothermal system at the Kolbeinsey Ridge, North of Iceland, we were able to obtain the coccoid-shaped *Nanoarchaeum equitans*, which represents a novel kingdom of Archaea (Huber et al. 2002). With a cell diameter of only 400 nm, it is the smallest living organism known. Cells grow attached to the surface of a specific crenarchaeal host, a new member of the genus *Ignicoccus* (Fig. 10). Owing to their unusual ss rRNA sequence, members of *Nanoarchaeum equitans* remained undetectable by commonly used “universal” primers in ecological studies based on the polymerase chain reaction. *Nanoarchaeum equitans* represents the deepest branching lineage within the Archaea, suggesting that the *Nanoarchaeota* diverged very early in the history of life (Fig. 5). The genome of *Nanoarchaeum equitans* harbours some big surprises (Waters et al. 2003): with only 490,885 bp, it is the smallest microbial genome known to date and also the most compact, with 95% of the DNA predicted to encode proteins or stable RNAs. This genome encodes the complete machinery for information processing and repair, but lacks genes for lipid, cofactor, amino acid, and nucleotide biosynthesis. The limited biosynthetic and catabolic capacity of *Nanoarchaeum equitans* suggests that its symbiotic relationship to its *Ignicoccus* host may be parasitic, making it the only known archaeal parasite. Unlike the small genomes of bacterial parasites however, that are undergoing reductive evolution, the small genome of *Nanoarchaeum equitans* has very few pseudogenes and a well equipped DNA recombination system. In contrast to modern parasites, no free-living relatives of *Nanoarchaeum* could be detected, so far. Therefore, it may be a

very ancient parasite. At present, we are still far away from a deeper understanding of the *Nanoarchaeum-Ig-nicoccus hospitalis*-relationship and further investigations are required. The *Nanoarchaeota* are inhabiting hot environments world-wide. Their discovery suggests that further major groups of microbes may still be out so far unrecognized, which are waiting for their isolation in order to tell us more about the evolution and origins of life.

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