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Mini-review

# The phylum Nanoarchaeota: Present knowledge and future perspectives of a unique form of life

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## Abstract

The “Nanoarchaeota” are a novel archaeal phylum, forming a unique, deep branch in the 16S rRNA based phylogenetic tree of life. “*Nanoarchaeum equitans*”, the first cultivated representative, is a hyperthermophilic, anaerobic nano-sized coccus with a genome size of about 490 kb. Growth occurs only in coculture with a new chemolithoautotrophic *Ignicoccus* species.

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## 1. Introduction

Thus far, the domain of the Archaea has consisted of three major branches: the Crenarchaeota (harboring three validly described orders with 24 genera), the Euryarchaeota (10 orders, and more than 50 different genera), and the so-called “Korarchaeota”, a group which has only been postulated on PCR-based amplification of 16S rRNA genes from environmental DNA [1,32]. The phylogeny of the Archaea is usually determined by sequence comparisons of their 16S rRNA genes; but similar tree topologies are obtained by sequence comparisons of 23S rRNAs and of various proteins [10,13,19,21,22].

The cultivated Archaea are highly diverse in their physiological characteristics: from strict chemolithoautotrophs, gaining energy by sulfur, sulfate, nitrate, nitrite, carbonate and oxygen respiration to obligate heterotrophs, from mesophiles to hyperthermophiles, from obligate aerobes to strict anaerobes, and from fresh water organisms to hypersaline life forms [15,16,30]. A few Archaea are even known to be living inside the cytoplasm of eukaryotes [7,24].

The morphology of the Archaea described today shows great diversity as well: short to long rods with a length of

1  $\mu\text{m}$  up to more than 100  $\mu\text{m}$ , straight or slightly bent, sometimes even branched (e.g., *Thermoproteales* [34]). The cell diameter is usually around 0.4 to 1  $\mu\text{m}$ , but some organisms are only 0.17  $\mu\text{m}$  wide (e.g., *Thermofilum* [29,33]). Coccoid cells are regular to highly lobed spheres (e.g., *Sulfolobus* [6], *Pyrodictium* [27], *Pyrolobus* [4]) with diameters between 0.5  $\mu\text{m}$  up to about 15  $\mu\text{m}$  (*Staphylothermus* [11]). The cell wall of most Crenarchaeota consists of an S-layer, usually a single (glyco-)protein species, arranged in the form of a two-dimensional crystalline sheet [3,9,20,28]; *Ignicoccus* cells are a notable exception: they are the first Archaea to possess an outer membrane [25].

Very recently, from a sample of a marine hydrothermal system near Iceland, a coculture of a new *Ignicoccus* strain and small archaeal cocci was obtained. The small cocci turned out to represent the first member of a novel archaeal phylum, the “Nanoarchaeota” [18]. This article summarizes the present knowledge on their phylogeny, physiology and ultrastructure, and discusses open questions and future perspectives.

## 2. Isolation, morphology and ultrastructure

On a diving excursion to the hydrothermal system at Kolbeinsey ridge north of Iceland samples of hot rocks and gravel were taken at a depth of about 106 m [12]. From enrichment cultures, a new member of the crenarchaeal genus

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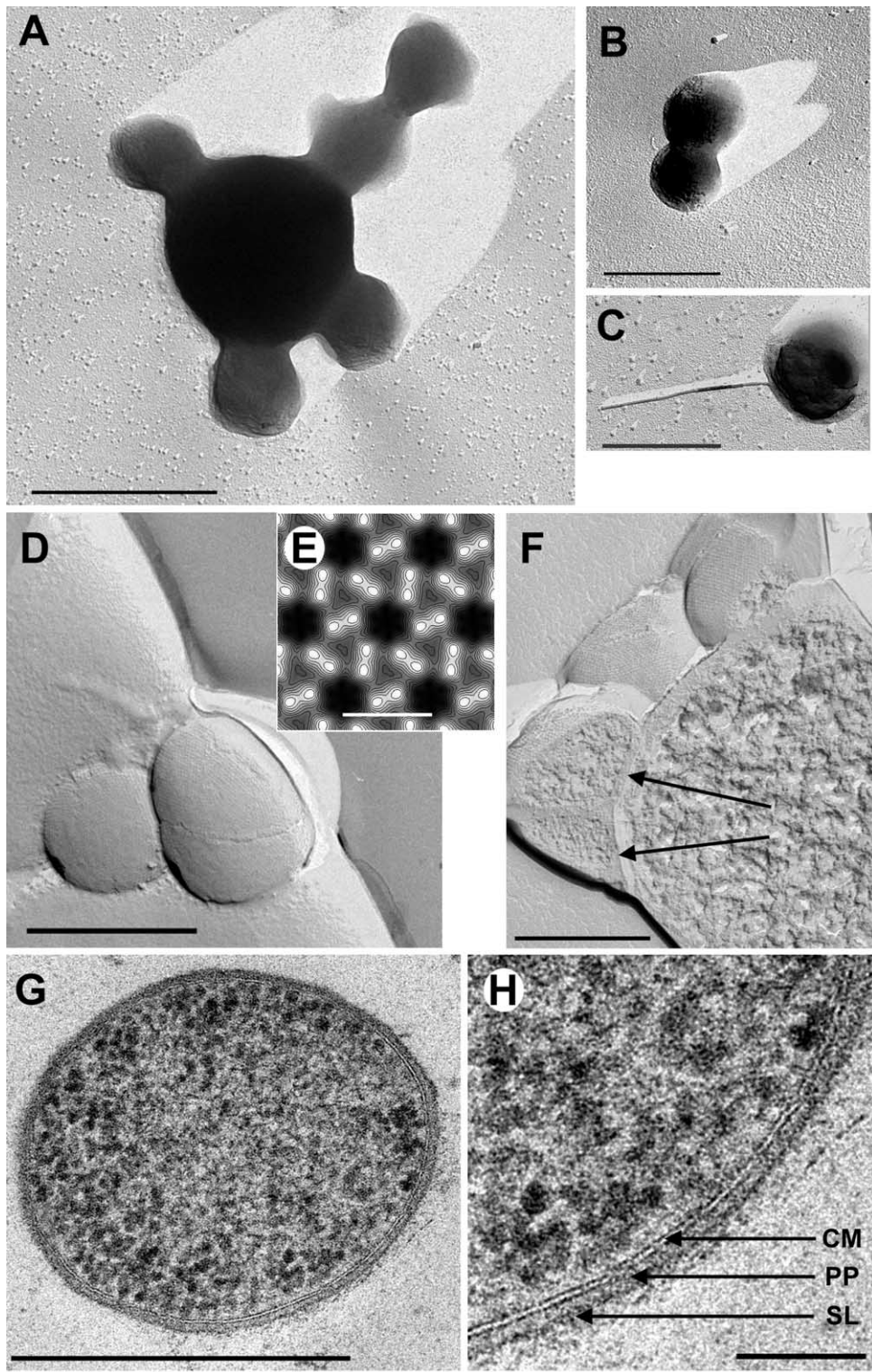


Fig. 1. Transmission electron micrographs of “*N. equitans*”. (A) Five cells of “*N. equitans*”, attached on the surface of the (central) *Ignicoccus* cell. Platinum shadowed. Bar: 1  $\mu\text{m}$ . (B) “*N. equitans*” cell in the late phase of division. Platinum shadowed. Bar: 0.5  $\mu\text{m}$ . (C) “*N. equitans*” cell with an extracellular appendage, possibly a flagellum. Platinum shadowed. Bar: 0.5  $\mu\text{m}$ . (D) Two freeze-etched “*N. equitans*” cells, attached on the surface of two *Ignicoccus* cells. The “*N. equitans*” cell on the right is in the early phase of division. Bar: 0.5  $\mu\text{m}$ . (E) Surface relief reconstruction of the “*N. equitans*” cell. Bar: 15 nm. (F) Several freeze-etched cells and one freeze-fractured cell (in the phase of division) of “*N. equitans*” (left), attached to a (freeze-fractured) *Ignicoccus* cell (right). The arrows point to the attachment site of the outer membrane of *Ignicoccus* and the S-layer of “*N. equitans*”. Bar: 0.5  $\mu\text{m}$ . (G, H) Ultrathin section of a “*N. equitans*” cell, prepared by high-pressure freezing, freeze-substitution, and embedding in Epon. (G) Single cell. Bar: 0.5  $\mu\text{m}$ . (H) At higher magnification; CM: cytoplasmic membrane; PP periplasm; SL: S-layer. Bar: 0.1  $\mu\text{m}$ .

*Ignicoccus* was isolated, *Ignicoccus* sp. strain KIN4/I. By careful inspection of these cultures using phase contrast light microscopy, these cells were occasionally but consistently seen to possess very small, coccoid “appendages” on their outer surface. They turned out to consist of tiny cocci, with an extremely small cell diameter of about 400 nm. They are attached to the surface of the *Ignicoccus* sp. KIN4/I cells, and therefore, the name “*Nanoarchaeum equitans*” has been proposed [18]. They occur singly, in pairs (Fig. 1A), or sometimes 10 or more cells per *Ignicoccus* cell. Single cells and cells in pairs are occasionally found freely in suspensions (Fig. 1B, C). Transmission electron micrographs show the “*N. equitans*” cells to be regular cocci with a diameter of only about 0.35–0.5  $\mu\text{m}$ , occasionally with a single extracellular appendage, possibly a flagellum (Fig. 1C). In freeze-etched preparations, the “*N. equitans*” cells are seen to be in direct attachment with the outer membrane of *Ignicoccus* cells (Fig. 1D, F). Their cell surface is covered by an S-layer with a lattice constant of 15 nm. Surface relief reconstruction [2] reveals hexagonal symmetry (Fig. 1E); the arrangement of the subunits can be interpreted to be in the form of protein dimers, arranged on the two-fold symmetry axis. This has to be preliminary, as long as a detailed three-dimensional reconstruction of the isolated S-layer is not available. Ultrathin sections (Fig. 1G, H) reveal the architecture of the cell envelope of this archaeum: the cytoplasmic membrane is about 8 nm wide; the outermost ‘sheath’ is the S-layer, in sections visible as a zig-zag shaped thin line; jointly, cytoplasmic membrane and S-layer delimit the periplasmic space, which is about 20 nm wide.

### 3. Physiology

As a major characteristic “*N. equitans*” only grows in coculture with *Ignicoccus* strain KIN4/I. Both can be cultivated in half strength SME medium [17] by anaerobic incubation at 90 °C in the presence of  $\text{S}^{\circ}$  without organic components and an atmosphere of  $\text{H}_2$  and  $\text{CO}_2$  (80:20, v/v, 300 kPa). As metabolic end product of the coculture,  $\text{H}_2\text{S}$  is formed, similar to the pure *Ignicoccus* culture. We have not yet found any further metabolic product in the coculture. The final cell density in serum bottles of both “*N. equitans*” and *Ignicoccus* strain KIN4/I is about  $3 \times 10^7$  cells  $\text{ml}^{-1}$ . In such a culture, about 50% of the *Ignicoccus* cells are occupied with one to more than ten “*N. equitans*” cells.

Since growth of “*N. equitans*” depends on propagating *Ignicoccus* cells (strain KIN4/I), quite similar growth parameters are observed for both organisms. Growth of the coculture occurs under strictly anaerobic conditions between 70 and 98 °C. Minimal doubling times (70 min for *Ignicoccus*, 45 min for *N. equitans*) are obtained at 90 °C, pH 6, and at salt concentrations of 2% NaCl. By adjusting the gassing rate to 30  $\text{l min}^{-1}$  ( $\text{H}_2:\text{CO}_2 = 80:20$ ), the final cell density of “*N. equitans*” can be raised about 10-fold in enamel protected fermentors (e.g., 300 l volume), while the cell den-

sity of *Ignicoccus* remains unchanged. This procedure efficiently removes  $\text{H}_2\text{S}$  and improves the supply of molecular hydrogen. During the late exponential growth phase of such a culture, about 80% of the “*N. equitans*” cells detach from their host cells and occur freely in suspension.

Several observations let us suggest that “*N. equitans*” and *Ignicoccus* KIN4/I exhibit a symbiotic mode of life: (i) isolated “*N. equitans*” cells do not grow on cell homogenates of *Ignicoccus*, they require an actively growing *Ignicoccus* culture; (ii) attempts to grow “*N. equitans*”, separated from *Ignicoccus* by a semipermeable membrane, failed so far; therefore, a direct cell–cell contact with the host organism appears to be a prerequisite for growth of “*N. equitans*”; (iii) a wide variety of single and complex organic compounds was tested but did not significantly increase the final cell density of “*N. equitans*”. Nevertheless, even a parasitic lifestyle of “*N. equitans*” relative to *Ignicoccus* cannot be excluded at present. However, this coculture demonstrates that close relationships (symbiosis or parasitism) occur not only between members of different domains [7,24], but even within the Archaea.

### 4. Phylogeny

At present, the phylum “Nanoarchaeota” harbors one genus with one species: “*N. equitans*” [18]. Besides this organism, three further “nanoarchaeotal” 16S rDNA sequences, obtained from environmental DNAs from different high temperature biotopes are known (LPC33, OP9, and CU1; see below) [14].

The sequences of the 16S rRNA gene of “*N. equitans*” and of the nanoarchaeotal environmental rDNAs are unique. They harbor many base exchanges even in so-called “highly conserved regions” usually employed as primer targets for PCR [18]. Due to this great divergence, cells of “*N. equitans*” do not stain by fluorescence in situ hybridization (FISH) using 16S rRNA-targeted oligonucleotide probes directed against Crenarchaeota and Euryarchaeota (e.g., EURY498R, CREN499R [8]). However, using oligonucleotide probes based on the “*N. equitans*” sequence resulted in a positive fluorescence signal [18].

In contrast to the unique primary structure of the 16S rRNA molecule, its secondary structure exhibits the typical features of an archaeal 16S rRNA [18]. Nevertheless, 16S rDNA sequence comparisons reveal that the phylum “Nanoarchaeota” represents an isolated deep lineage within the Archaea distinct from the Crenarchaeota, Euryarchaeota and the “Korarchaeota” (Fig. 2). Within the phylum, “*N. equitans*” and the three environmental 16S rDNA sequences exhibit phylogenetic similarities between 83 and 100% [14]. The sequence from the East Pacific Rise (LPC33) turned out to be identical to the sequence of “*N. equitans*”, while the others exhibit new primary structures. The three sequences consistently group together in phylogenetic

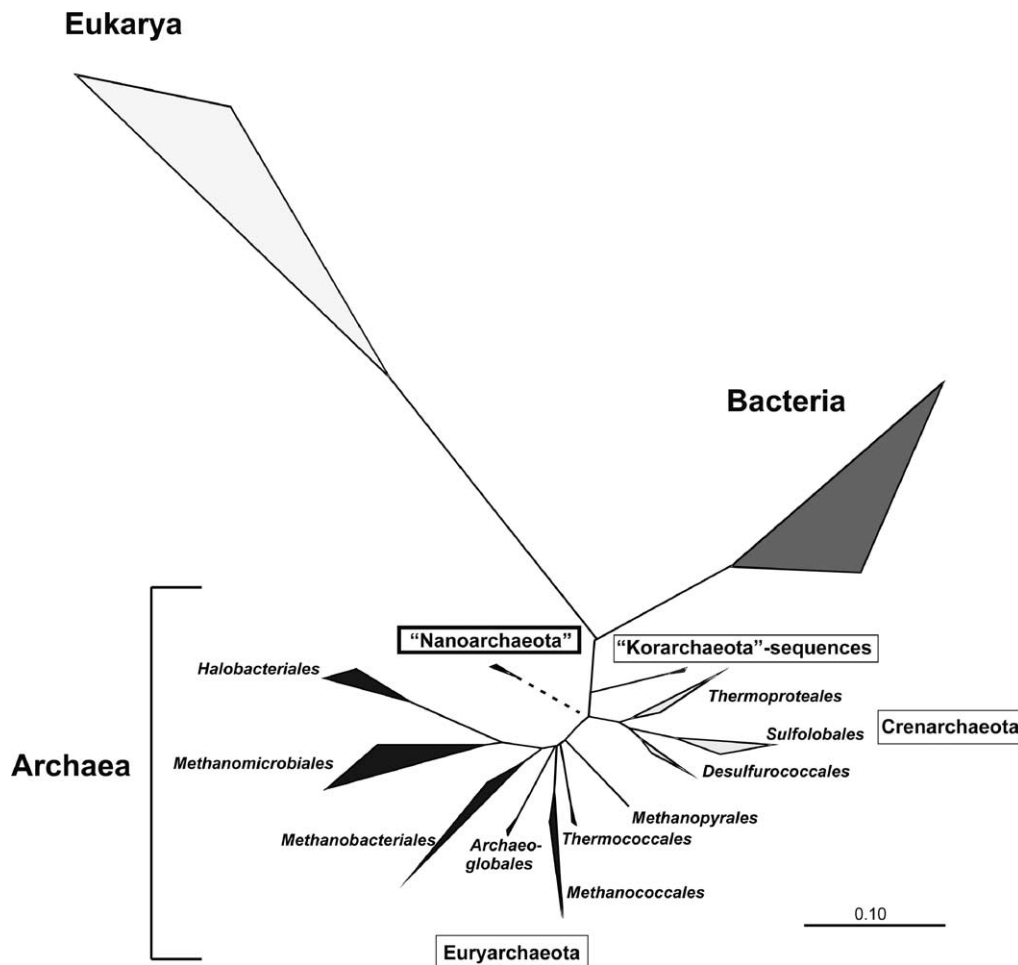


Fig. 2. Phylogenetic tree based on 16S rRNA sequence comparisons. The tree was calculated using the maximum likelihood (FastDNAmI) program, embedded in the ARB package [21]. The interrupted line for the “Nanoarchaeota” outlines the uncertain position of the branching point.

analyses with high bootstrap support (98 to 100%), independently of the calculation algorithm applied (distance matrix: neighbor joining, Fitch–Margoliash algorithm, maximum parsimony and maximum-likelihood methods). The phylogenetic similarities to all other archaeal species are between 67 and 80% [14], demonstrating that the phylum is well defined by 16S rRNA gene sequence comparisons. However, the placement of the “Nanoarchaeota” branch within the Archaea is still an open question. Although they always branch off very deeply, the position of the lineage within the phylogenetic tree varies significantly, depending on the analytical method used. This correlates with low and insignificant bootstrap values for the branching point of the “Nanoarchaeota”. Phylogenetic investigations based on analyses of the 23S rRNA sequence of “*N. equitans*” resulted in similar tree topologies and were consistent with the 16S rRNA trees (Hohn and Huber, unpublished). New organisms and/or nanoarchaeotal sequences may help to stabilize the rRNA-based trees. In addition, the phylogenetic position of the “Nanoarchaeota” can be addressed using sequence comparisons of proteins, when the whole genome sequence will be available.

## 5. Molecular characteristics

The genome size of “*N. equitans*” was determined after digestion of its chromosome with restriction endonucleases and separation of the resulting fragments by pulsed-field gel electrophoresis (PFGE) (Fig. 3). From the sizes of the fragments obtained, the genome of “*N. equitans*” can be estimated to be about 490 kb, and therefore it is the smallest archaeal genome known today. This size was verified by the results of whole genome sequencing, which was carried out recently at DIVERSA Inc., San Diego, CA, USA. Details on the study of the genome will be available in the near future. Small genome sizes are very common among obligate parasitic and symbiotic bacteria (like *Mycoplasma genitalium*, *Buchnera* sp., or *Rickettsia prowazekii*) and have developed by the elimination of unneeded genes in combination with a restricted uptake of new genes by lateral gene transfer [23]. Possibly, the genome of “*N. equitans*” has also undergone size reduction during the adaptation to its host. On the other hand it is assumed that the genomes of the first microorganisms were small [31] and therefore a small genome may also be an ancient one. Due to the

deep phylogenetic branching position of “*N. equitans*” and its hyperthermophilic life style it is tempting to speculate that its small genome size is rather a primitive than a derived feature. Answers to this question may be given after detailed analyses of its genome content.

EF-1 $\alpha$  (= EF-Tu in Bacteria) is ubiquitously present in all organisms. A short oligopeptide fragment of its GDP binding domain has been used previously to distinguish Eu-

karya and Crenarchaeota from Euryarchaeota and Bacteria [26]. An insertion of an 11-amino-acids fragment into this domain is only present in Eukarya and Crenarchaeota. It is missing in Euryarchaeota and Bacteria; instead these groups possess a segment of four to seven amino acids. Therefore, analysis of the GDP binding domain of “*N. equitans*” provides a further phylogenetic marker for the nanoarchaeotal phylum.

After PCR amplification, the corresponding gene fragment of “*N. equitans*” (as well as that of *Ignicoccus* KIN4/I) was cloned and sequenced. We compared the predicted amino acid sequence with those of other Archaea, Bacteria and Eukarya. As expected, *Ignicoccus* strain KIN4/I harbors the 11 amino acid segment (indicated in Fig. 4), while it is not present in “*N. equitans*”. Instead, it possesses a 4-amino-acid segment (GVQE, Fig. 4) (Hohn and Huber, unpublished), a feature shared by Euryarchaeota and Bacteria. Therefore, it can be speculated that this structure is characteristic for the whole phylum, although the verification has to await the isolation of further members of this group.

### 6. Biotopes and distribution

“*N. equitans*” was obtained from a sample of hot rocks taken at the Kolbeinsey Ridge, north of Iceland [18], a hydrothermal system located at the subpolar Mid-Atlantic Ridge at a depth of around 106 m [12]. Further “nanoarchaeotal” 16S rRNA genes were obtained from environmental DNAs from a submarine high temperature biotope at the East Pacific Rise (black smoker fragment, original pH 6.5), and from two continental solfataric areas with low ionic strength: Obsidian Pool, Yellowstone National Park, USA

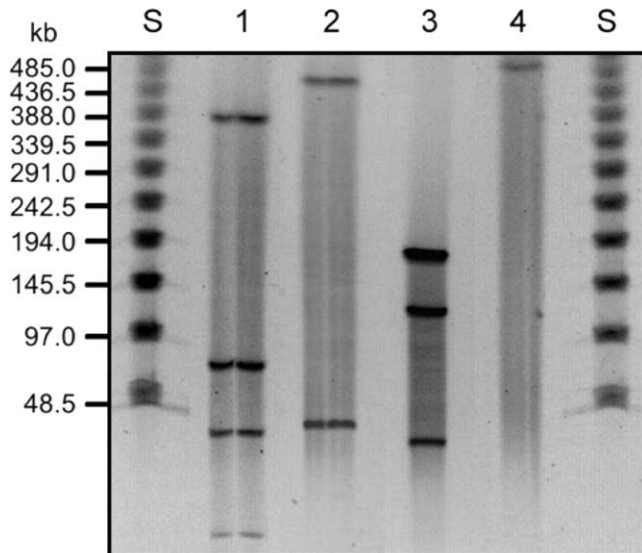


Fig. 3. Pulsed-field gel electrophoresis (PFGE) separation of digested and undigested chromosomal DNA of “*Nanoarchaeum equitans*”. Lane 1: *Bss*HIII digestion; lane 2: *Asc*I digestion; lane 3: *Not*I digestion; lane 4: undigested chromosomal DNA; S = Lambda Ladder PFG Marker (New England Biolabs). From the digestions the genome size can be estimated to be 480 kb (370 + 70 + 30 + 10 kb, *Bss*HIII), 490 kb (455 + 35 kb, *Asc*I), 490 kb (175 + 170 + 120 + 25 kb, *Not*I) and 490 kb (undigested).

	10	20	30	40	
<i>Saccharomyces cerevisiae</i>	KNMITGTSQADCAILLIAGGV	--GEFEAGISKDG	-----	-----	QTREH
<i>Homo sapiens</i>	KNMITGTSQADCAVLIVAAGV	--GEFEAGISKNG	-----	-----	QTREH
<i>Drosophila melanogaster</i>	KNMITGTSQADCAVQIDAAGT	--GEFEAGISKND	-----	-----	QTREH
<i>Rattus rattus</i>	KNMITGTSQADCAVLIVAAGV	--GEFEAGISKNG	-----	-----	QTREH
<i>Desulfurococcus mobilis</i>	KNMITGASQADAAILVVSARK	--GEFEAGMSAEG	-----	-----	QTREH
<i>Pyrodicticum occultum</i>	----GASQADAAILVVSARK	--GEFEAGMSAEG	-----	-----	-----
<i>Aeropyrum pernix</i>	KNMITGASQADAAILVVSARK	--GEFEAGMSTEG	-----	-----	QTREH
<i>Sulfolobus solfataricus</i>	KNMITGASQADAAILVVSARK	--GEFEAGMSVEG	-----	-----	QTREH
<i>Sulfolobus acidocaldarius</i>	KNMITGASQADAAILVVSARK	--GEFEAGMSAEG	-----	-----	QTREH
<i>Staphylothermus marinus</i>	KNMITGASQADAAILVVSARK	--GEFEAGMSPEG	-----	-----	QTREH
<i>Ignicoccus</i> strain Kin4/I	KNMITGASQADAAILVVSARP	--GEFEAGMSAEG	-----	-----	QTREH
<i>Pyrobaculum aerophilum</i>	KNMIVGASQADAALFVISARP	--GEFEAAGPQG	-----	-----	QGREH
<i>Thermoplasma acidophilum</i>	KNMITGTSQADAAILVISARE	-----	-----	-----	GEGVME--QTREH
<i>Methanococcus jannaschii</i>	KNMITGASQADA AVLVDVND	-----	-----	-----	AKTGIQP--QTREH
<i>Methanococcus vanniellii</i>	KNMITGASQADA AVLVDVDD	-----	-----	-----	AKSGIQP--QTREH
<i>Archaeoglobus fulgidus</i>	KNMITGASQADA AVLMDVVE	-----	-----	-----	KVQPP--QTREH
<i>Thermococcus celer</i>	KNMITGASQADA AVLVAATD	-----	-----	-----	GVMP--QTKHE
<i>Pyrococcus abyssi</i>	KNMITGASQADA AVLVAATD	-----	-----	-----	GVMP--QTKHE
<i>Pyrococcus horikoshii</i>	KNMITGASQADA AVLVAATD	-----	-----	-----	GVMP--QTKHE
<i>Pyrococcus woesei</i>	KNMITGASQADA AVLVAATD	-----	-----	-----	GVMP--QTKHE
<i>Methanothermobacter thermoautotr.</i>	KNMITGASQADA AVLVAATD	-----	-----	-----	GVMP--QTKHE
<i>Halobacterium salinarum</i>	KNMITGASQADNA VLVAATD	-----	-----	-----	GVAP--QTKHE
“ <i>Nanoarchaeum equitans</i> ”	KNMITGASQADA AVLVAADD	-----	-----	-----	GVQE--QTKHE
<i>Thermotoga maritima</i>	KNMITGAAQMDGAILVVAATD	-----	-----	-----	GPMP--QTKHE
<i>Escherichia coli</i>	KNMITGAAQMDGAILVVAATD	-----	-----	-----	GPMP--QTKHE

Fig. 4. Comparison of EF-1 $\alpha$  and EF-Tu amino acid sequences of Eukarya, Crenarchaeota, Euryarchaeota, Bacteria, and “Nanoarchaeota”. Eleven amino acid segments are characteristic for Eukarya and Crenarchaeota; four amino acid segments are characteristic for Bacteria and Euryarchaeota and are also present in “*N. equitans*”.

(original temperature 80 °C, pH 6.0) and Caldera Uzon, Kamchatka, Russia (original temperature 85 °C, pH 5.5) [14]. These results document a broad distribution of members of the “Nanoarchaeota” in high temperature biotopes in the deep sea, in shallow marine areas and in solfataric fields on land. Moreover, the habitats are located on different continents, suggesting a world-wide distribution for the “Nanoarchaeota”.

## 7. Conclusions and outlook

The discovery of “*Nanoarchaeum equitans*” and the related 16S rDNA sequences has significantly increased our knowledge of the diversity of Archaea and of hyperthermophiles. Although our knowledge of physiological and molecular characteristics of “*N. equitans*” is still preliminary, it can be expected that detailed studies on the interaction with its host will give general insights into the organization of the symbiotic/parasitic mode of life, into interspecies exchange of biomolecules, and into regulation phenomena. From the analysis of its genome, which is under way, further knowledge on its metabolism and molecular biochemistry can be expected. However, experimental limitations may occur based on the fact that “*N. equitans*” can only be grown in coculture with a *Ignicoccus* strain. In addition, it cannot be plated (yet), and mutants are not available.

The cell and genome size of “*N. equitans*” are close to the theoretical limits of a living cell (e.g., the volume of a “*N. equitans*” cell is less than 1% of the volume of an *E. coli* cell). At present, an interesting open question is whether “*N. equitans*” is a still primitive form of life (due to its high growth temperature and anaerobic mode of life), or if it is a highly derived descendant of an already known group with a reduced (degenerated or streamlined) genome, based on its symbiotic/parasitic mode of life [5,18]. Analysis of the “*N. equitans*” genome and the isolation of further members of this phylum may give insights into this question. However, from the current information, it is unclear whether the new “Nanoarchaeota” sequences represent small and symbiotic/parasitic organisms similar to “*N. equitans*” and which organisms might serve as possible hosts.

The isolation of “*N. equitans*” also has consequences upon our interpretation of environmental studies based on PCR-amplified (16S rRNA) genes and the diversity of life on earth. Studies with so-called universal PCR primers necessarily restrict our view to those organisms whose rRNA sequence is already known or at least very similar to cultivated organisms. It cannot, however, highlight organisms whose rRNA is so different that it is not recognized or amplified by these primers, as was the case with “*N. equitans*”. Therefore, the discovery of “*N. equitans*” promises that the diversity of microbial life on earth may be much greater than it was formerly expected.

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