A Novel Kingdom of Parasitic Archaea

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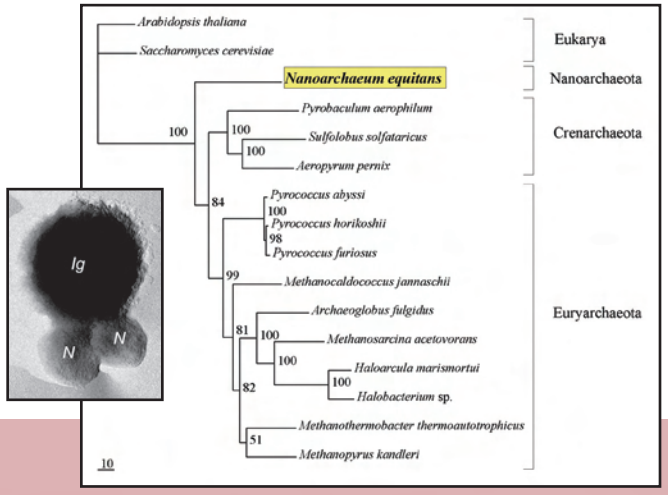

ABSTRACT

A nano-sized obligate symbiont obtained from a submarine hydrothermal system north of Iceland represents a novel kingdom of *Archaea*—the Nanoarchaeota. Cells of *Nanoarchaeum equitans* are only 400 nm in diameter, resulting in a cell volume of less than 1% of an *Escherichia coli* cell. They grow attached to the surface of a new *Ignicoccus* species under strict anaerobic conditions at temperatures between 75°C and 98°C. In contrast to the *Ignicoccus* host cell, *N. equitans* possesses an S-layer. The small subunit SSU rRNA gene exhibits a unique sequence characterized by base exchanges even in segments previously thought to be identical for all organisms ("universal" signatures). *N. equitans* harbours a genome of only 490 kb, one of the smallest genomes known so far. The analysis of its gene content reveals only very limited biosynthetic and metabolic capacities, indicating that the symbiotic relationship to *Ignicoccus* is parasitic. Comparison of the membrane lipids of *N. equitans* and its host *Ignicoccus* reveals that both organisms harboured qualitatively identical lipids suggesting that the *N. equitans* lipids are synthesized by the *Ignicoccus* host. Unlike many small genome bacterial parasites, *N. equitans* has few pseudogenes or regions of non-coding DNA. Examination of environmental DNAs from terrestrial hot springs at Yellowstone National Park; Uzon Caldeira, Russia; and from an abyssal vent system at the East Pacific Rise resulted in the finding of further novel nanoarchaeotal SSU rRNA gene sequences, demonstrating great diversity and worldwide distribution of the Nanoarchaeota which had been completely overlooked, so far.

Key Words

genome sequencing
hyperthermophilic
Nanoarchaeota
parasite
phylogeny
16S rDNA

1.0 INTRODUCTION

More than 30 years ago, in his pioneering work in Yellowstone National Park, Tom Brock discovered *Sulfolobus acidocaldarius,* the first organism with a growth optimum at 70–75°C and exhibiting an upper growth temperature of 75–85°C (Brock et al. 1972). Its aerobic metabolism and extreme thermal adaptation initially suggested a uniquely specialized, highly derived prokaryote (Castenholz 1979). *S. acidocaldarius* was subsequently reclassified by Carl Woese as a member of the newly discovered *Archaea* (Woese et al. 1978). Since then, a variety of almost exclusively anaerobic microorganisms with optimal growth temperatures above 80°C and upper growth temperatures of more than 100°C have been isolated (Blöchl et al. 1995). These organisms are now designated by the new term "hyperthermophiles" (Stetter 1989). Hyperthermophiles had been discovered in terrestrial high temperature environments like solfataras, hot springs, smoldering coal refuse piles, and deep, geothermally-heated rocks. In addition, submarine high temperature environments like active sea mounts, volcanically heated sediments, hot vents, and black smokers harbour hyperthermophiles which are adapted to the high salinity of sea water (Stetter 1999). Hyperthermophiles occur within both prokaryotic domains—*Bacteria* and *Archaea* (Woese et al. 1990). In phylogenetic trees based on (SSU) rRNA sequence comparisons, hyperthermophiles represent all the short deep lineages, forming a cluster around the root (Stetter 1995). This feature, along with their hot ancient biotopes, suggests that hyperthermophiles are still rather primitive. So far, all cultivated archaea including hyperthermophiles belong to the kingdoms, or phyla, of Crenarchaeota and Euryarchaeota. Ten years ago, based only on environmental DNA sequences obtained from Yellowstone's Obsidian Pool, a third archaeal kingdom—the Korarchaeota—was postulated (Barns et al. 1996). Here we describe the discovery, cultivation, and properties of novel hyperthermophilic parasites which belong to a further, so far unknown, kingdom of *Archaea* and which occur in submarine and terrestrial high temperature areas including Yellowstone.

2.0 MATERIAL AND METHODS

2.1 Sampling and Enrichment

High-temperature submarine sandy sediments, venting water, and materials from black smokers were obtained from the Kolbeinsey Ridge, north of Iceland, located at the shallow subpolar Mid-Atlantic Ridge (depth around 106 m; Fricke et al. 1989); the East Pacific Rise (lat 9°N, long 104°W; depth approximately 2500 m); the Guaymas Basin (between lat 20°49′N, long 109°06′W and lat 27°01′N, long 111°24′W; depth approximately 2000 m); and Vulcano Island, Italy (depth up to 15 m; original temperatures up to 102°C). In addition, sediments and spring waters from terrestrial solfataric areas were taken at Yellowstone National Park (Obsidian Pool); Kamchatka Peninsula, Russia (Uzon Caldeira, Karymsky Volcano, Puchino hot springs); Atacama Desert, Chile (Termas de Jurasi, Termas de Pollequere, Puchuldiza geysers, Tatio volcanic area); and Lihir Island, Papua New Guinea. The original temperatures of these samples ranged from 70°C to 98°C with pH from 5.5 to 6.5. All samples were brought to our laboratory without temperature control and stored at 4°C in our sample collection for one month to four years prior to the investigations. Enrichment attempts were carried out in ½ SME medium, described by Stetter (1982) and modified by Blöchl et al. (1997). Anaerobic incubation occurred at 90°C in the presence of S^0, H_2 : CO_2 (80 : 20, v/v, 300 kPa) without organic components.

2.2 Cultivation and Isolation Procedure

For the cultivation of *Ignicoccus* sp. strain KIN4/I and *Nanoarchaeum equitans*, strictly anaerobic ½ SME medium adjusted to pH 5.5 was used. The organisms were grown routinely in 120 mL serum bottles containing 20 mL medium pressurized with H_2 : CO_2 (80 : 20, v/v; 250 kPa). Incubation was carried out at 90°C under shaking (100 rpm). Large-scale fermentations were carried out in a 300 L enamel-protected fermentor (HTE, Bioengineering) at 90°C under stirring (100 rpm) and gassing with H_2 : CO_2 (80 : 20, v/v). Defined co-cultures of *N. equitans* and *Ignicoccus* sp. strain KIN4/I were obtained by addition of single cells of *N. equitans* into pure cultures of *Ignicoccus* and by cloning of

Ignicoccus cells with one *N. equitans* cell attached employing optical tweezers (Huber et al. 1995).

2.3 DNA Isolation, PCR Amplifications, and Phylogenetic Analyses

DNA from more than 30 original samples were prepared as described (Hohn et al. 2002). PCR amplifications were performed using the Nanoarchaeota-specific primers 7mcF, 518mcF, 1116mcR, and 1511mcR (Hohn et al. 2002). The amplified 16S rRNA gene fragments were purified and cloned. Twenty-five to 30 clones were randomly chosen for plasmid preparation which was performed by a standard procedure (Sambrook et al. 1989). The inserts were re-amplified from the plasmids, digested separately with two restriction endonucleases, and compared on agarose gels by amplified rDNA restriction analysis, or ARDRA (Laguerre et al. 1994). The 16S rDNA clones obtained from the primer combinations were sequenced with the primers 7mcF, 344aF, 518mcF, 1116mcR, 1119aR, and 1511mcR. For phylogenetic analyses, an alignment of about 11,000 full and partial primary sequences available in public databases (ARB project; Ludwig and Strunk 2001) was used. The new 16S rRNA gene sequences were fitted in the corresponding trees by using the automated tools of the ARB software package. Distance matrix (neighbour joining, Fitch-Margoliash algorithm; Fitch and Margoliash 1967), each with Jukes-Cantor correction (Jukes and Cantor 1969), maximum parsimony, and maximum-likelihood (fastDNAml) methods were carried out as implemented in the ARB package.

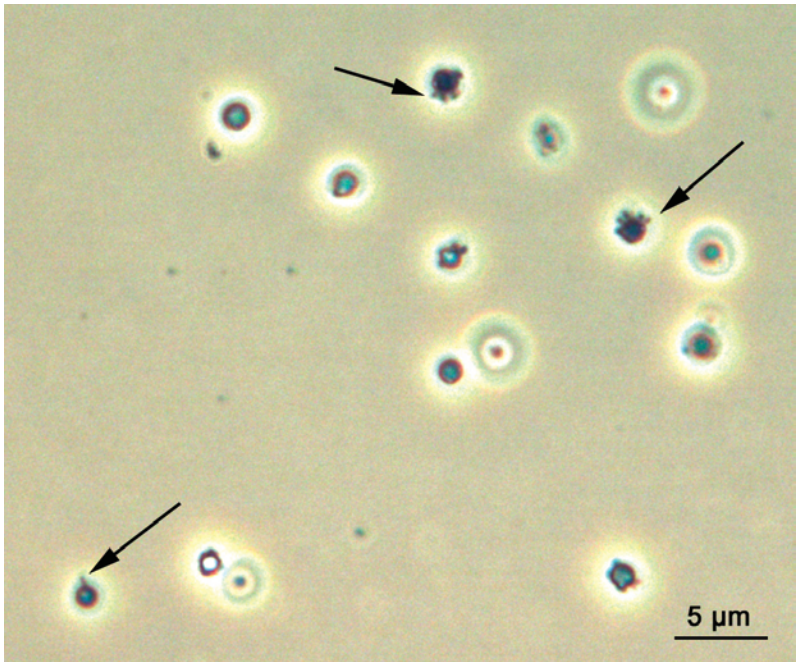

Figure 1. Coculture of *Nanoarchaeum equitans* and *Ignicoccus* sp. KIN4/1. Phase contrast micrograph; Bar 5 µm. Arrows: attached *N. equitans* cells (examples).

3.0 RESULTS AND DISCUSSION

3.1 Discovery, Morphology, and Ultrastructure

To enrich novel hyperthermophilic microorganisms, samples of hot rocks and gravel from the hydrothermal system at the Kolbeinsey Ridge north of Iceland were taken at a depth of 106 m (Huber et al. 2002). In these experiments a new member of the crenarchaeal genus *Ignicoccus* was obtained (strain KIN4/I). Cells of this new strain regularly possessed very small, coccoid "appendages" on their outer surface which turned out to consist of very tiny cocci, about 400 nm in diameter. The tiny cells were attached directly to the surface of the *Ignicoccus* cells, appearing to ride there. Therefore, we named the novel organism *Nanoarchaeum equitans*, the "riding primeval dwarf" (Huber et al. 2002). Cells occurred singly, in pairs, and up to about 30 cells per *Ignicoccus* cell (**Figures 1 and 2A**). In the stationary growth phase, many cells were also found free in suspension. The cell volume of *N. equitans* was only about 1% of an *E. coli* cell. In freeze-etched preparations, a direct attachment of the *N. equitans* S-layer to the outer membrane of the *Ignicoccus* cells became evident (**Figure 2B**). The *N. equitans* S-layer exhibited a lattice constant of 15 nm and a hexagonal symmetry of its subunits (**Figure 2C**). The reconstruction of the surface

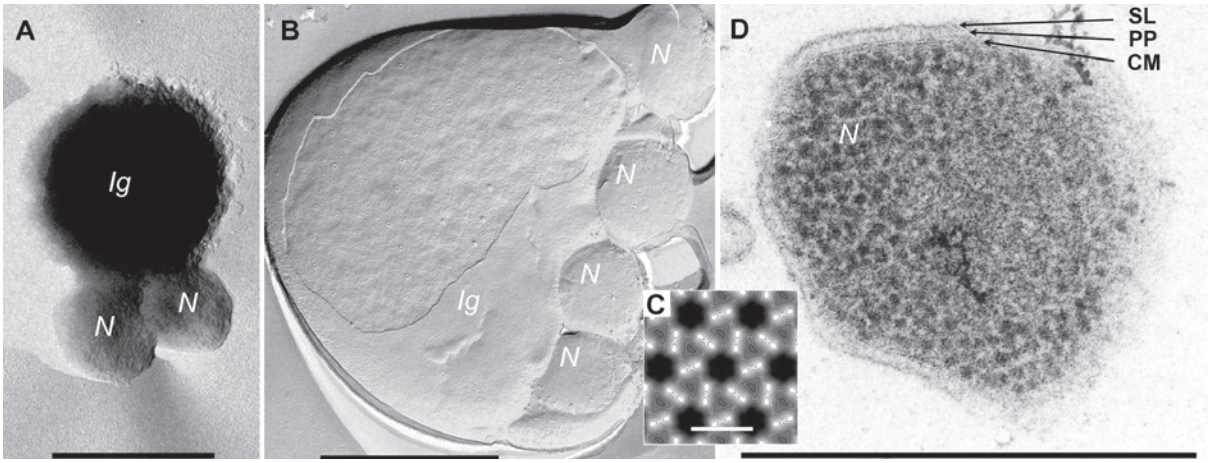

Figure 2. Transmission electron micrographs of *Nanoarchaeum equitans*. A. Two cells of *N. equitans* (N), attached on the surface of the (central) *Ignicoccus* cell (Ig). Platinum shadowed. Bar: 1 µm. **B.** Freeze-etched cell of *Ignicoccus* and attached cells of *N. equitans* on the surface. Bar: 1 µm. **C.** Surface relief reconstruction of *N. equitans*. Dark: cavity; Bright: elevation. Bar: 15 nm. **D.** Ultrathin section of a *N. equitans* cell, prepared by high-pressure freezing, freeze-substitution, and embedding in Epon. Single cell. CM: cytoplasmic membrane; PP: periplasm; SL: S-layer. Bar: 0.5 µm. (Figure 2b from Huber et al. 2002; Figure 2c from Huber et al. 2003)

relief shows that the proteins are arranged in the form of dimers on the two-fold symmetry axis. Ultrathin sections (**Figure 2D**) revealed the architecture of the cell envelope of this archaeon: a cytoplasmic membrane about 8 nm wide; a periplasmic space about 20 nm wide; and as the outermost sheath, the S-layer, in sections visible as a thin zigzag line.

3.2 Physiology

We found that *N. equitans* could only be cultivated in coculture with *Ignicoccus* strain KIN4/I where it required an actively growing *Ignicoccus* culture (Huber et al. 2002). It could not be grown on cell homogenates of its host. As a consequence, quite similar growth parameters were observed for both organisms. They grew under strictly anaerobic conditions between 70°C and 98°C. Optimal doubling times were obtained at 90°C, pH 6, and at salt concentrations of 2% NaCl in the presence of S^0 without organic components and an atmosphere of H_2 and CO_2 (80 : 20, v/v, 300 kPa). Similar to the pure *Ignicoccus* culture, H_2S was formed as a metabolic end-product of the coculture (Huber et al. 2002). The final cell density in serum bottles of both organisms was about 3 x 10^7 cells mL^{-1}. By adjusting the gassing rate to 30 L min^{-1} (H_2 : CO_2 = 80 : 20), a procedure which efficiently removes H_2S, the final cell concentration of *N. equitans* could be raised about tenfold in the fermentor (e.g., 300 L volume), while that of *Ignicoccus* remained unchanged. During the late exponential growth phase, about 80% of the *N. equitans* cells detached from their host cells and occurred freely in suspension (Huber et al. 2003).

3.3 Phylogeny

The phylogenetic relationships of *N. equitans* were investigated by SSU rRNA sequence comparisons (Woese et al. 1990; Ludwig and Strunk 2001; Huber et al. 2002). Total DNA of the coculture was extracted and SSU rRNA genes were amplified by PCR employing primers addressed to highly conserved regions. Surprisingly, however, only the *Ignicoccus* SSU rRNA gene sequence was amplified, even with primers considered general for all organisms (universal primers). In a more direct approach, by examination of the coculture-derived DNA for SSU rRNA genes by Southern blot hybridization, two different hybridization signals became visible, indicating two non-identical SSU rRNA genes which belonged to *Ignicoccus* and *N. equitans*. After cloning and sequencing, the *N. equitans* sequence proved truly unique, harbouring many base exchanges even in so-called highly conserved regions, which are usually employed

Table 1. **Comparison of standard SSU rRNA gene PCR primers with the corresponding sequences of the Nanoarchaeota.**

Primer	**8aF**	**344aF**
Sequence	TCY GGT TGA TCC TGC C	CGG CGY GCA SCA GGC GCG AA
N. equitans sequence	TCC CGT TGA TCC TGC G	CGG GGC GCA CCA GGG GCG AA
OP9 / CU1 sequence	n.d.	CGG GAT GCA CCA GGG GCG AA
Primer	**519uF**	**934aR**
Sequence	CAG CMG CCG CGG TAA TAC	GTG CYC CCC CGC CAA TTC CT
N. equitans sequence	CAG CMG CCG CGG GAA CAC	GTG CTC CCC CGC CTA TTC CT
OP9 / CU1 sequence	CAG TCG CCA CGG GAA TAC	GTG CCC CCC CGC CTA TTC CT
Primer	**1044aF**	**1119aR**
Sequence	GAG AGG WGG TGC ATG GCC G	GGY RSG GGT CTC GCT CGT T
N. equitans sequence	GAG AGG AGG TGC ATG GCC G	GGC GCG GGT CTC GCC TGT T
OP9 / CU1 sequence	GAG AGG AGG TGC ATG GCT G	GGT GCG GGT CGC GCT CGT T
Primer	**1406uR**	**1512uR**
Sequence	ACG GGC GGT GTG TRC AA	ACG GHT ACC TTG TTA CGA CTT
N. equitans sequence	ACG GGC GGT GAG TGC AA	ACG GCT ACC TTG TGT CGA CTT
OP9 / CU1 sequence	ACG GGC GGT GAG AGC AA	n.d.

Green: specific for all Nanoarchaeota
Blue: specific for *N. equitans*
Red: specific for clone sequences (CU1 and OP9)
Source: Hohn et al. (2002)

as primer targets in the PCR reaction (**Table 1**). Comparison of the new sequence with those of other microorganisms revealed that *N. equitans* is a member of the domain *Archaea*, which was further confirmed by the presence of several archaea-specific helices in its secondary structure (Huber et al. 2002). The sequence identities to the Crenarchaeota, Euryarchaeota, and Korarchaeota were between 0.7 and 0.8, in the same range as those between these three kingdoms of *Archaea*. Therefore, *N. equitans* represents a new archaeal kingdom which we named the Nanoarchaeota.

In SSU rRNA phylogenetic trees that were founded on distance matrix (neighbour joining, Fitch-Margoliash algorithm; Fitch and Margoliash 1967), maximum parsimony, and maximum likelihood methods, *N. equitans* represented an isolated, deeply branching lineage. However, due to its unique SSU rRNA, a large variation of its branching point challenged by insignificant bootstrap values became evident (Huber et al. 2002). Sequencing of the *N. equitans* genome, followed by concatenation and alignment of the amino acid sequences of 35 ribosomal proteins (Wolf et al. 2001; Matte-Tailliez et al. 2002; Waters et al. 2003) shed light on its phylogenetic relationship among the archaea. *N. equitans* was placed with high bootstrap support at the most deeply branching position within *Archaea* in maximum parsimony, maximum likelihood, and Bayesian trees (**Figure 3**), suggesting that the Nanoarchaeota diverged early within *Archaea* (Waters et al. 2003). Due to missing information about their ribosomes, the Korarchaeota could not be considered in this procedure.

3.4 Genome Analysis

The genome of *N. equitans* (GenBank accession no. AACL01000000) pointed to a parasitic lifestyle. It consisted of a single, circular chromosome of only 490,885 bp and had an average G + C content of 31.6%. A total of 552 coding DNA sequences (CDS) with an average length of 827 bp could be identified (Waters et al. 2003). Despite having the smallest genome of a cellular organism sequenced so far, *N. equitans* exhibited the highest gene density with CDS and stable RNA sequences covering about 95% of the genome. Functional roles could be assigned to two-thirds of the annotated genes. Gene clusters (putative operons), although less common in *Archaea* than in *Bacteria*, are rarely conserved between *N. equitans* and other archaea.

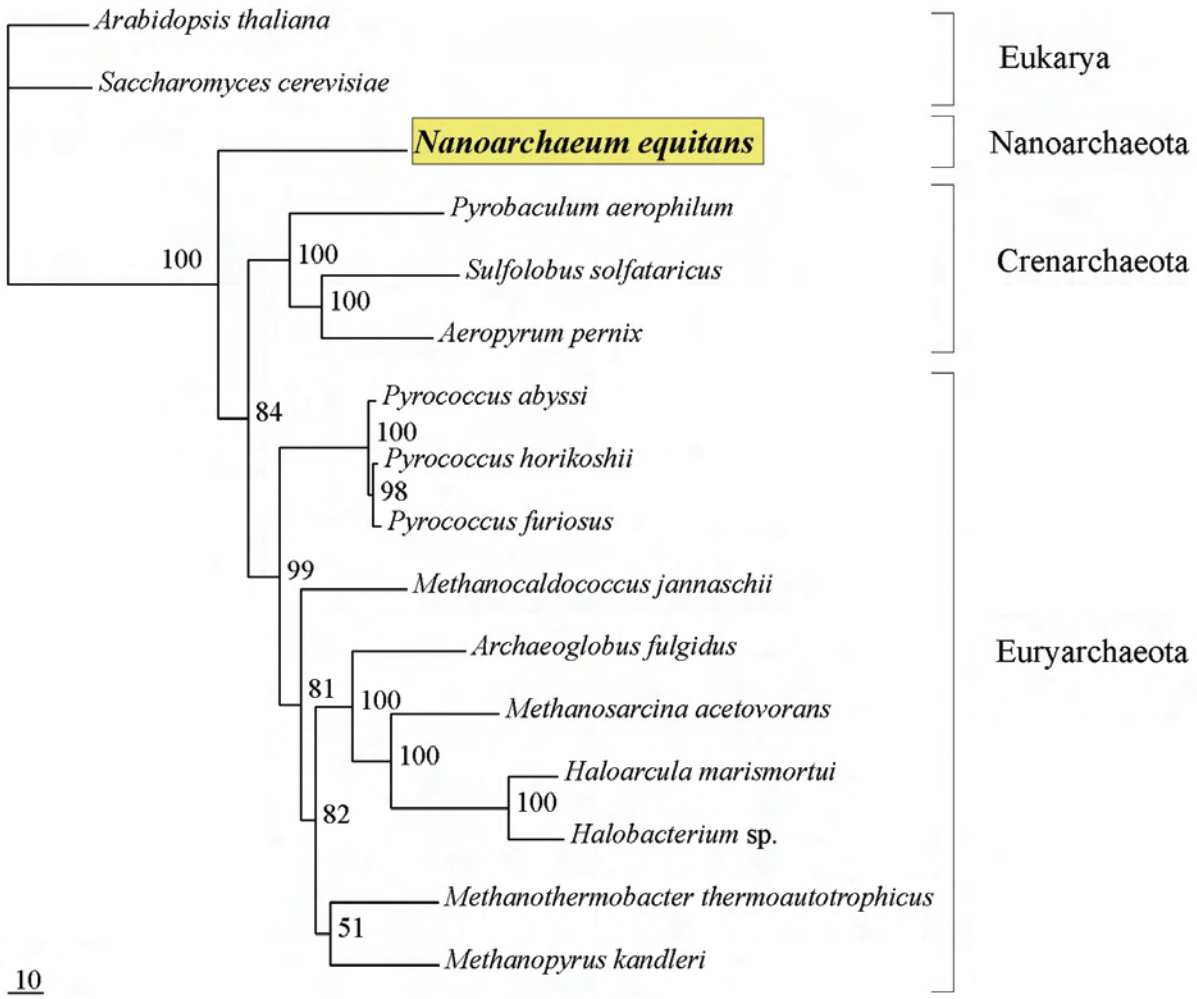

Figure 3. Phylogenetic position of *N. equitans* within the *Archaea*. The tree is based on 35 concatenated ribosomal protein amino acid sequences. Numbers indicate percentage of bootstrap resamplings. Tree calculated by the maximum likelihood method with PROML from the PHYLIP package (Felsenstein 2001). (From Waters et al. 2003).

Even ribosomal proteins that are clustered together in bacterial, euryarchaeal, and crenarchaeal genomes were dispersed in the *N. equitans* genome (Waters et al. 2003). Unlike its *Ignicoccus* host, which gains energy by hydrogen-sulfur autotrophy (Fischer et al. 1983), *N. equitans* had no genes to support a chemolithoautotrophic physiology. However, the genome encoded a branched-chain amino acid aminotransferase and glutamate dehydrogenase for amino acid oxidative deamination. There were also a limited number of enzymes that could catalyze electron transfer reactions. The genome encoded five subunits of an archaeal A_1A_o-type ATP synthase which was much simpler than the prototypical nine-subunit ATPase. In view of its small genome size, it was not surprising that *N. equitans* lacked the metabolic capacity to synthesize many cell components. Almost all genes that are required for the *de novo* biosyntheses of nucleotides, amino acids, cofactors, and lipids were missing. This organism also

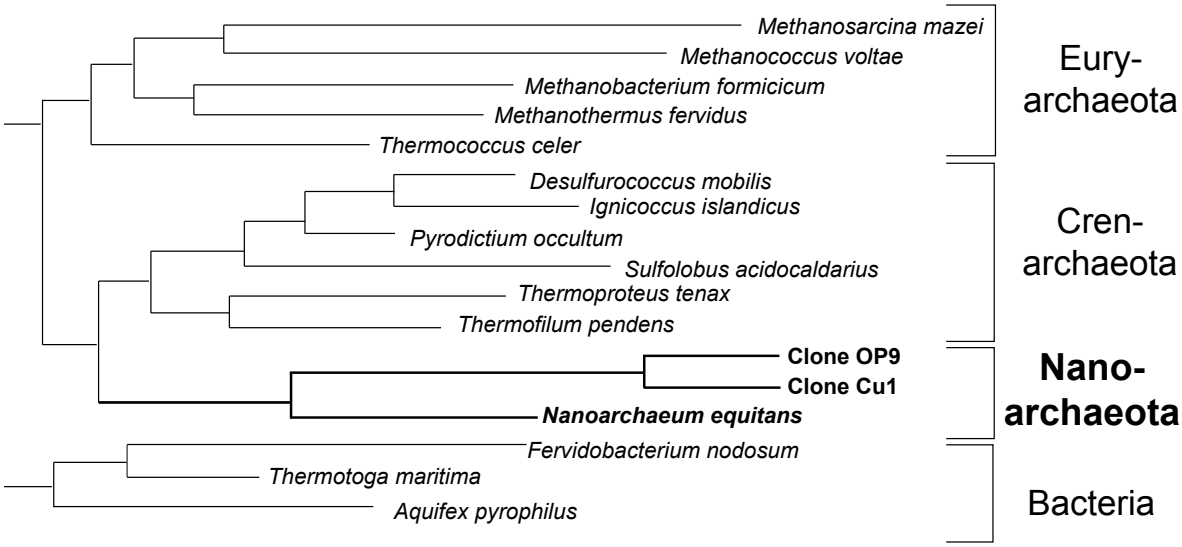

Figure 4. Phylogenetic tree based on 16S rRNA sequence comparisons. The tree was calculated using the maximum likelihood (FastDNAml) program, embedded in the ARB package (Ludwig and Strunk 2001).

lacked genes for glycolysis/gluconeogenesis, the pentose phosphate pathway, the tricarboxylic acid cycle, and other known pathways for carbon assimilation. This absence of metabolic capacity necessitates the transport of most cellular metabolites from the *Ignicoccus* host. Although seven putative transporters had been identified in this genome, this set of membrane proteins appeared insufficient to import all the metabolites required by *N. equitans* (Waters et al. 2003). The lipids of *N. equitans* may be acquired directly from its host by employing vesicles formed at the cytoplasmic membrane of *Ignicoccus* (Huber et al. 2000).

In contrast to its paucity of metabolic genes, *N. equitans* harboured a reasonably complete set of components for information processing (replication, transcription, and translation) and completion of the cell cycle (Waters et al. 2003). An unusual characteristic of the *N. equitans* genome was the high number of split genes whose gene product is encoded by two unlinked CDS. The split sites for most of these genes lay between functional domains of the encoded proteins. Therefore, it seems likely that the two separated genes are expressed to form subunits of a functional enzyme. This could be confirmed for the genes for the two subunits of alanyl-tRNA synthetase which were separated by half of the *N. equitans* chromosome (Waters et al. 2003). The *N. equitans* reverse gyrase was split into two distinct genes encoding a helicase and a topoisomerase domain. Reverse gyrase appeared to be the fusion product of a helicase and a topoisomerase domain, and catalyzes positive supercoil formation in DNA (Kikuchi and Asai 1984; Krah et al. 1996; Forterre 2002). Because this complex enzyme is present only in hyperthermophiles, it was concluded that hyperthermophily appeared secondarily in the evolution of life (Forterre et al. 2000). In light of the presence of independent helicase and topoisomerase domains in the deep-rooted *N. equitans*, the evolution of hyperthermophily may have been a very early event in agreement with the view of a hot primeval Earth (Stetter 1997). Assuming that multi-domain proteins evolved from the fusion of simple domains, then split genes could reflect the multi-subunit ancestral state of the proteins (Doolittle 1978; Gilbert 1987).

In contrast to many obligate bacterial parasites with small genomes, *N. equitans* harboured a full set of DNA repair and recombination enzymes which may be required to repair DNA damage most likely occurring in its high-temperature habitat (Waters et al. 2003). It

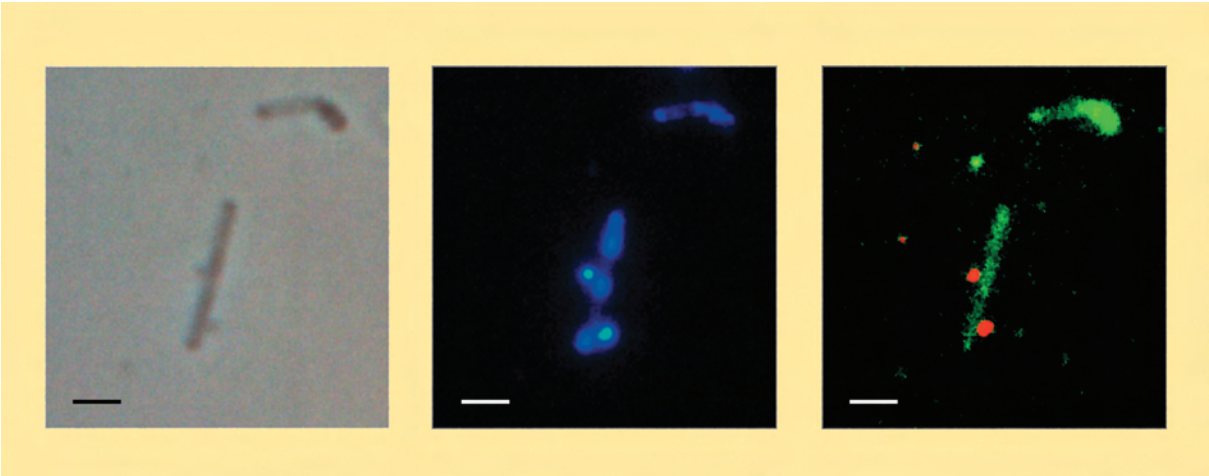

Figure 5. Identification of nanoarchaeal cells attached to rod-shaped archaeal cell in the enrichment culture CU1, derived from a Kamchatka terrestrial hot spring. Left: Phase contrast micrograph; middle: DNA-specific stain (DAPI); Right: FISH stain, Red = Nanoarchaea-specific probe (position 915-935); Green = Crenarchaea-specific probe (position 499-515).

is remarkable that many organisms with small genomes have lost recombination/repair enzymes even though such losses have significant negative consequences by fixing of deleterious mutations and formation of many (non-functional) pseudogenes (Moran 1996; Tamas et al. 2002). The genetic conservation of split genes, along with the paucity of pseudogenes and a minimum of noncoding DNA (below 5%) suggests that the *N. equitans* genome is evolutionary stable compared with many bacterial parasites (Waters et al. 2003).

3.5 Distribution of the Nanoarchaeota

To investigate the distribution of the Nanoarchaeota, samples from further marine and from terrestrial high temperature environments were screened for the presence of nanoarchaeal SSU rRNA gene sequences. These genes were amplified by PCR employing Nanoarchaea-specific primers followed by sequencing (Hohn et al. 2002). From fragments of an abyssal black smoker situated at the East Pacific Rise (lat 9°N, long 104°W, Alvin dive no. 3072, sample LPC33), a nanoarchaeal sequence could be obtained which was identical with the original sequence of *N. equitans* which had been isolated from the subpolar Mid-Atlantic Ridge close to Kolbeinsey (Fricke et al. 1989). With the same PCR-based procedure, for the first time in terrestrial high temperature environments Nanoarchaeota-sequences were found in Yellowstone and in Kamchatka (Hohn et al. 2002). Samples had been taken from Yellowstone's Obsidian Pool (OP9: water and black sediment, 80°C, pH 6.0) and from a small hot waterhole with blackish, vigorously gassed water at Uzon Caldeira (CU1: water and black sediment, 83°C, pH 5.5). By distance matrix analyses, both terrestrial sequences (OP9 and CU1) revealed about 93% similarity among each other and only about 83% similarity to the (marine) *N. equitans* and LPC33 sequences, as shown by the calculation-based dendrogram (**Figure 4**). Therefore, the novel terrestrial sequences reveal great phylogenetic diversity among the Nanoarchaeota, with members of different taxonomic groups thriving within different biotopes. Initial microscopic inspection of the samples obtained from Obsidian Pool revealed tiny cocci, about the size of *N. equitans* attached to the surface of *Pyrobaculum*-shaped rods that may represent these novel nanoarchaeotes. From sample CU1, an enrichment culture was recently obtained in which tiny cocci, identified by FISH-staining as nanoarchaeotes, were attached to rod-shaped cells (**Figure 5**). Therefore, the terrestrial nanoarchaeotes may also have

a parasitic lifestyle. These results demonstrate a worldwide distribution of the Nanoarchaeota within high temperature biotopes, from the deep sea to shallow submarine areas to terrestrial solfataras.

4.0 CONCLUSIONS

In light of the discovery of the Nanoarchaeota, environmental diversity studies solely based on PCR-amplified (SSU rRNA) genes should be interpreted more cautiously. Studies with so-called universal PCR primers restrict our view to organisms whose SSU rRNA target sequence is already known or at least similar to cultivated species. They cannot, however, highlight members of unknown groups with rRNAs too different to be recognized by the primers applied. The discovery of the Nanoarchaeota demonstrates much greater diversity of microbial life on Earth than formerly expected; further, it suggests the existence of exciting life forms, still unrecognizable by PCR gene amplification, that await discovery and cultivation.

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