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A new symbiotic nanoarchaeote (*Candidatus* Nanoclepta minutus) and its host (*Zestosphaera tikiterensis* gen. nov., sp. nov.) from a New Zealand hot spring

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ABSTRACT

Three thermophilic *Nanoarchaeota-Crenarchaeota* symbiotic systems have been described. We obtained another stable anaerobic enrichment culture at 80 °C, pH 6.0 from a New Zealand hot spring. The nanoarchaeote (Ncl-1) and its host (NZ3^T) were isolated in co-culture and their genomes assembled. The small (~200 nm) flagellated cocci were often attached to larger cocci. Based on 16S rRNA gene similarity (88.4%) and average amino acid identity (52%), Ncl-1 is closely related to *Candidatus* Nanopusillus acidilobi. Their genomes both encode for archaeal flagella and partial glycolysis and gluconeogenesis pathways, but lack ATP synthase genes. Like *Nanoarchaeum equitans*, Ncl-1 has a CRISPR-Cas system. Ncl-1 also relies on its crenarchaeotal host for most of its biosynthetic needs. The host NZ3^T was isolated and grows on proteinaceous substrates but not on sugars, alcohols, or fatty acids. NZ3^T requires thiosulfate and grows best at 82 °C, pH 6.0. NZ3^T is most closely related to the *Desulfurococcaceae, Ignisphaera aggregans* (~92% 16S rRNA gene sequence similarity, 45% AAI). Based on phylogenetic, physiological and genomic data, Ncl-1 and NZ3^T represent novel genera in the *Nanoarchaeota* and the *Desulfurococcaceae*, respectively, with the proposed names *Candidatus* Nanoclepta minutus and *Zestosphaera tikiterensis* gen. nov., sp. nov., type strain NZ3^T (=DSMZ 107634^T = OCM 1213^T).

Introduction

Despite a ubiquitous distribution and a diversity that may parallel that of the *Bacteria*, the *Archaea* still remain the most unexplored of life's domains. Many new lineages without cultivated representatives have been discovered through metagenomic and single cell genomic approaches, giving rise to a significant increase in new archaeal phyla and divisions [4,7,83,89,99]. One exception is the *Nanoarchaeota*, a phylum with only two representatives in culture [35,106]. The first isolate, *Nanoarchaeum equitans*, was obtained from a shallow marine hydrothermal site off Iceland and forms an obligate parasitic relationship with its autotrophic archaeal host, *Ignicoccus hospitalis* [35]. With its highly reduced genome, *N. equitans* lacks most primary biosynthetic functions and has to acquire many metabolic precursors from its host [105]. The second nanoarchaeote in culture was obtained from an acidic hot spring in Yellowstone National Park (YNP) [106]. This terrestrial representative, *Candidatus* Nanopusillus acidilobi, is also an obligate symbiont, and its host is an acidophile and heterotroph, *Acidilobus* sp. 7A. Like its marine relative, *Cand.* Nps. acidilobi shows extensive genome reduction and requires its host for most primary biosynthetic and energetic needs, yet it has retained some potential for glycolysis and gluconeogenesis, expresses archaeal flagella genes and lacks a membrane ATP synthase complex [106]. The differences between the two cultivated nanoarchaeotes point to how diverse symbiotic associations may be within the *Nanoarchaeota*. Additional terrestrial *Nanoarchaeota* symbiotic systems were characterized based on single cell sequencing from geothermal acidic pools in YNP [68,83]. Even though these environmental nanoarchaeote genomes were very similar to *Cand.* Nps. acidilobi, their implied hosts were quite

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dissimilar and more closely related to *Sulfolobus* than *Acidilobus* [68,83].

Based on 16S rRNA gene surveys, *Nanoarchaeota* have been detected globally in terrestrial and marine hot springs. These include many sites in YNP [16,34,67,68], Kamchatka [15,34], Chile [14], Central Asia [14], China [14], New Zealand [14], and deep-sea vents in the Atlantic [24] and Pacific oceans [26,34,63,69]. These different nanoarchaeotes form phylogenetic clades that can be separated somewhat by geographical region. It is therefore likely that as more members of this group and their hosts are characterized, a much better understanding of their biology and evolutionary history will emerge. Here, we report on the enrichment and isolation of a third nanoarchaeote in stable co-culture with its host. This nanoarchaeote was obtained from a terrestrial hot spring in New Zealand and more closely resembles its relatives from YNP than *N. equitans*. We also describe the isolation and characterization of the host, a novel genus in the *Desulfurococcaceae*. The *Nanoarchaeota*-host system highlights the need for formal recognition and classification for organisms that can be cultivated, but only in the presence of a symbiotic partner.

Materials and methods

Sample site and culturing conditions

Samples from "Cooking Pots" hot springs at Hell's Gate, Tikitere, New Zealand (38.0654°S, 176.3595°E) were inoculated into anaerobic enrichment media: Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) media #88 with (g/L): $Na_2O_3S_2{\cdot}5H_2O$, 2.0; NaOH, 0.16; yeast extract, 0.5; tryptone, 0.5; resazurin, 0.001; $FeCl_3{\cdot}6H_2O$, 0.004; $MnCl_2{\cdot}4H_20$, 0.00036; and 10 mL additional trace mineral solution DSMZ #141 and dispensed under N_2/CO_2 (80/20, v/v) and pH adjusted to 6.0–6.1. The enrichments were incubated at 80 °C for four days and checked by phase contrast microscopy. DNA was extracted using the Qiagen DNeasy Blood and Tissue DNA kit and the presence of *Nanoarchaeota* was checked using nanoarchaeotal-specific 16S rRNA gene primers [34]. Nanoarchaeotal-positive enrichment cultures were monitored over many transfers and maintained stably for over two years. A nanoarchaeote-host co-culture was isolated after several dilution-to-extinction transfers. Subsequently, the co-culture was transferred into, and routinely grown in, the above media with the following modifications: 0.5 g/L casein hydrolysate, 1.0 g/L yeast extract, 1.0 g/L tryptone, 0.0625 g/L L-cysteine-HCl and 10 mL/L glycerol.

Host isolation and 16S rRNA gene sequencing

The host was isolated by several series of dilution-to-extinction transfers in the described modified enrichment media. Genomic DNA was extracted as above, and the purity of the isolate was verified by PCR and sequencing of the 16S rRNA gene. Due to the presence of introns in the host 16S rRNA gene, two overlapping primer sets were utilized for sequencing: 4F (5′-TCCGGTTGATCCTGCCRG-3′), 907RN (5′-CCGYCAATTCMTTTRAGTTT-3′), 341FA (5′-CCTAYGGGGYGCASCAGGCG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR conditions consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 m 30 s, followed by a final elongation cycle at 72 °C for 7 min.

Physiological characterization of the host

Unless otherwise noted, all characterization studies were carried out in triplicate at 80–85 °C in sealed anaerobic Balch tubes. Cell density was monitored by phase contrast microscopy with a Petroff-Hausser counting chamber. Use of carbon sources was determined at a 0.1% (w/v) final concentration using a modified enrichment media with reduced yeast extract (0.01% w/v) and no casein hydrolysate, tryptone, or glycerol. Cultures were transferred at least once to minimize carry-over. The carbon sources that were tested included yeast extract, tryptone, casamino acids, gelatin, acetate, butyrate, cellulose, fructose, glucose, glycerol, mannose, propionate, sorbitol, ribose, starch, sucrose and xylose.

Alternate electron acceptor use was determined by replacing L-cysteine-HCl and thiosulfate in the enrichment media with a variety of electron acceptors at both 2.5 and 10 mM final concentrations. To prevent sublimation, elemental sulfur (2.5 and 10 mM) was added directly to media in individual Balch tubes prior to autoclaving at 105 °C for 1 h [25]. Potential electron acceptors included elemental sulfur, nitrate, thiosulfate, sulfite and L-cysteine-HCl. A positive growth response was only recorded after three consecutive transfers in the same medium.

To assess the pH range of isolate growth, enrichment media was supplemented with 25 mM MES (pH 5.5–6.0) and 25 mM MOPS (pH 6.3–6.8) [73]. The pH was adjusted to the desired range prior to autoclaving. Oxygen tolerance was tested by adding defined volumes (0–10%, v/v) of filtered oxygen directly to anaerobic culture tubes containing media with 10 mM thiosulfate but lacking L-cysteine HCl. Requirement for NaCl was determined using 0–1% NaCl (w/v).

Core and intact lipid preparation and analysis

Lipids were recovered using a modified Bligh and Dyer method [9], and polar head groups were removed by acidic methanolysis [74]. Core and intact lipids were analyzed using a modified version of the protocol described by Elling et al. [22]. Liquid chromatography–mass spectrometry (LCMS) was performed using a Shimadzu 8040-LCMS system equipped with a photodiode array detector and a triple quadrupole low resolution MS utilizing an ESI source for ionization and Q3 scanning from 300 to 2000 *m/z*. Lipids were identified based on comparison with published mass spectrometry data [65,109]. Additional methodologies for core and intact polar lipid analysis are available in the Supplementary information.

Electron microscopy

Negatively stained cells were prepared for transmission electron microscopy (TEM) by spotting 5 μL of culture onto formvar-carbon copper grids (Ted Pella, 400 mesh), and stained with 2% (w/v) uranyl acetate for 15 s and air dried. The micrographs were taken at acceleration voltage of 200 kV using a FEI TECNAI F-20 TEM.

qPCR assay

qPCR was performed using the QuantiTect® SYBR® Green PCR kit (Qiagen). Standards were developed with double-stranded 16S rRNA gene blocks (500 bp, gBlocks®, Integrated DNA Technologies) containing 165 bp regions flanking the target qPCR product. The qPCR primers were specific for the New Zealand nanoarchaeote: 515F-NZ (5′-GTGGGCAAGAGAGGTGG-3′) and 650R-NZ (5′-CGGCTCCTTCCGTCCCCTAG-3′) and were used at 0.2 μM final concentration. qPCR reaction conditions were as follows: 95 °C for 15 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s, followed by melt curve analysis from 55 to 95 °C.

Cell sorting and metagenomic sequencing

To obtain low diversity metagenomes, enrichment cultures containing nanoarchaeotes were sorted using flow cytometry as described previously [83]. To ensure recovery of nanoarchaeotes, multiple cells (5–10) were sorted into each well. After multiple displacement amplification (MDA), wells were screened using nanoarchaeotal-specific PCR primers [34,83]. Two wells with positive nanoarchaeotal amplification were selected for sequencing using the Illumina MiSeq. Adaptor sequences and low-quality regions were trimmed using Trim Galore [48]. After trimming, the two datasets yielded approximately 4.0 and 4.5 million paired-end reads, respectively.

Binning and assembly of metagenome

To maximize recovery of *Nanoarchaeota* and host genomes, raw reads from the two low diversity metagenomes were merged into one dataset. Reads were initially assembled using IDBA-UD v.1.1.0 [80,81]. Contigs ≥5 kb were binned using MaxBin v.1.4.5 [107]. To enhance data recovery, the combined dataset was re-assembled using the SPAdes genome assembler v.3.9.1-Linux [8]. Supervised binning of contigs ≥2.5 kb was performed using emergent self-organizing mapping (ESOM) based on tetranucleotide frequency [103]. Both the IDBA-UD and SPAdes assemblies were then merged and viewed in Geneious v.10.0.2 [42] for manual contig extension, curation, and identification of misassembled regions. Completeness and contamination of final draft genomes was assessed using CheckM [79].

Accessibility of data and biological material

Draft genomes of the New Zealand nanoarchaeote and its host are available in Genbank under accession numbers MWMI00000000 and NBVN00000000, respectively. 16S rRNA genes of the above strains have also been deposited under accession numbers MH298645 and MH252993. Project details are available in the NCBI BioProject database (PRJNA363059), and sampling metadata can be found in the NCBI BioSample database for the New Zealand nanoarchaeote (SAMN06250747) and its host (SAMN06648824). The nanoarchaeote-host co-culture has also been deposited in the Oregon Culture Collection of Methanogens (OCM 1214), and the host has been deposited in the OCM (OCM 1213^T) and the DSMZ (DSM 107634^T).

Functional annotation

After assembly, open reading frames were predicted and annotated using the Rapid Annotation and Subsystem Technology (RAST) server [5,11,77]. Additional annotation (Tables S1 and S2) was performed using the updated Archaeal Clusters of Orthologous Genes (arCOG) database [59,60]. As needed, annotations were cross-referenced using the NCBI Basic Local Alignment Search Tool (BLAST) [3] and the NCBI Conserved Domain Database [62,72]. Ribosomal RNAs were predicted with Infernal v.1.1.2 [71] and Rfam v.12.0 [70] and tRNAs were predicted with the tRNAscan-SE 2.0 web server [56]. Hydrogenases were predicted using the HydDB web server [98]. Circos ribbon plots [49] were used to compare overall genomic synteny between draft genomes and their closest described relatives. Pairwise average amino acid identity matrices were generated using the ANI/AAI Matrix Calculator [90].

Phylogenetic analysis

To assess the phylogenetic placement of the nanoarchaeote and its host, their 16S rRNA genes were aligned using the Geneious Aligner [42] and hypervariable regions were manually masked. Maximum-likelihood analysis was inferred with RAxML v.8.2.8 [100] under the GTRGAMMA model with 1000 replicate bootstrap trees, and trees were viewed using the Interactive Tree of Life [54]. 16S rRNA gene similarity was also assessed using EZBioCloud [44,108] and pairwise Geneious alignments. 16S rRNA gene introns identified in the host were analyzed for predicted secondary structure using the MFold web server [111]. Additional phylogenetic trees were built using a set of 16 ribosomal proteins [4] extracted with Phylosift v.1.0.1 [19]. After each set of ribosomal proteins was aligned in Muscle [20], alignments were concatenated in Geneious and maximum-likelihood analysis was performed using the PROTGAMMALG model as described. This model was also used to build single gene trees.

Results and discussion

Establishment of a stable nanoarchaeotal-host enrichment culture

Initial enrichments containing the New Zealand nanoarchaeote were complex (Fig. 1A) and often dominated by *Bacteria* belonging to the *Thermodesulfobacteriaceae* (97.47% similar in 16S rRNA gene sequence to *Thermodesulfobacterium geofontis* [32,44,108]). Other members of these enrichments included *Thermoanaerobacteraceae* related to *Caldanaerobacter*, *Dictyglomus* sp., *Fervidobacterium* sp., *Thermofilum* sp., the *Korarchaeota*, *Pyrobaculum* sp. and some unclassified *Thermoprotei* related to *Desulfurococcus* and *Ignisphaera*. Notably, some enrichments had up to 20% korarchaeotal sequences. Tracking the initial enrichment cultures by barcoded 16S rRNA gene sequencing (data not shown) revealed that *Thermodesulfobacteriaceae* dominated the cultures and reached stationary phase after 120 h. *Thermodesulfobacteriaceae* were also in highest relative abundance when the *Korarchaeota* were prevalent. Potentially, *Thermodesulfobacteriaceae* stimulated korarchaeotal growth by scavenging molecular hydrogen, as proposed by Elkins et al. [21]. The *Caldanaerobacter* sp. and *Fervidobacterium* sp. grew rapidly and reached stationary phase of growth after 24 h, while the *Dictyoglomus* sp. peaked after 72 h and the *Korarchaeota* after 144 h. The *Thermoprotei* and nanoarchaeotes were in very low relative sequence abundance and reached stationary growth phase around 72 h of incubation. From these enrichments, we successfully isolated pure cultures of strains related to *Dictyoglomus*, *Thermofilum*, *Thermodesulfobacterium*, *Caldanaerobacter*, *Desulfurococcus* and *Ignisphaera*.

Based on qPCR, the abundance of nanoarchaeotes in the enrichments increased with additional transfers, but then plateaued around 4–8 × 10^6 nanoarchaeotes/mL. Several early enrichments showed nanoarchaeotes attached to some, but not all, cocci (Fig. 1B). Nanoarchaeotal qPCR numbers closely tracked direct counts of the cocci in the cultures. Addition of sodium sulfide (0.005% w/v) did not stimulate nanoarchaeotal growth; starch (0.5% w/v) and the presence of hydrogen in the headspace inhibited growth. Highest nanoarchaeotal numbers were obtained between 80–85 °C although they were still detected at 60 °C and at 90 °C.

Early dilution-to-extinction series were not successful in isolating the New Zealand nanoarchaeote and its host. No nanoarchaeotes could be detected in initial serial dilutions greater than 10^{-3}. Possibly the dilutions were outgrown by some other members of the enrichment, particularly the fastidious *Thermodesulfobacteriaceae*. Furthermore, attempts to obtain colonies with nanoarchaeotes on solid media roll tubes (Gelrite™, Research Products International) were unsuccessful. As repeated attempts to isolate pure nanoarchaeotal-host cultures by these methods were not successful, additional approaches to stimulate nanoarchaeotal growth and obtain less complex enrichments were used. A series of

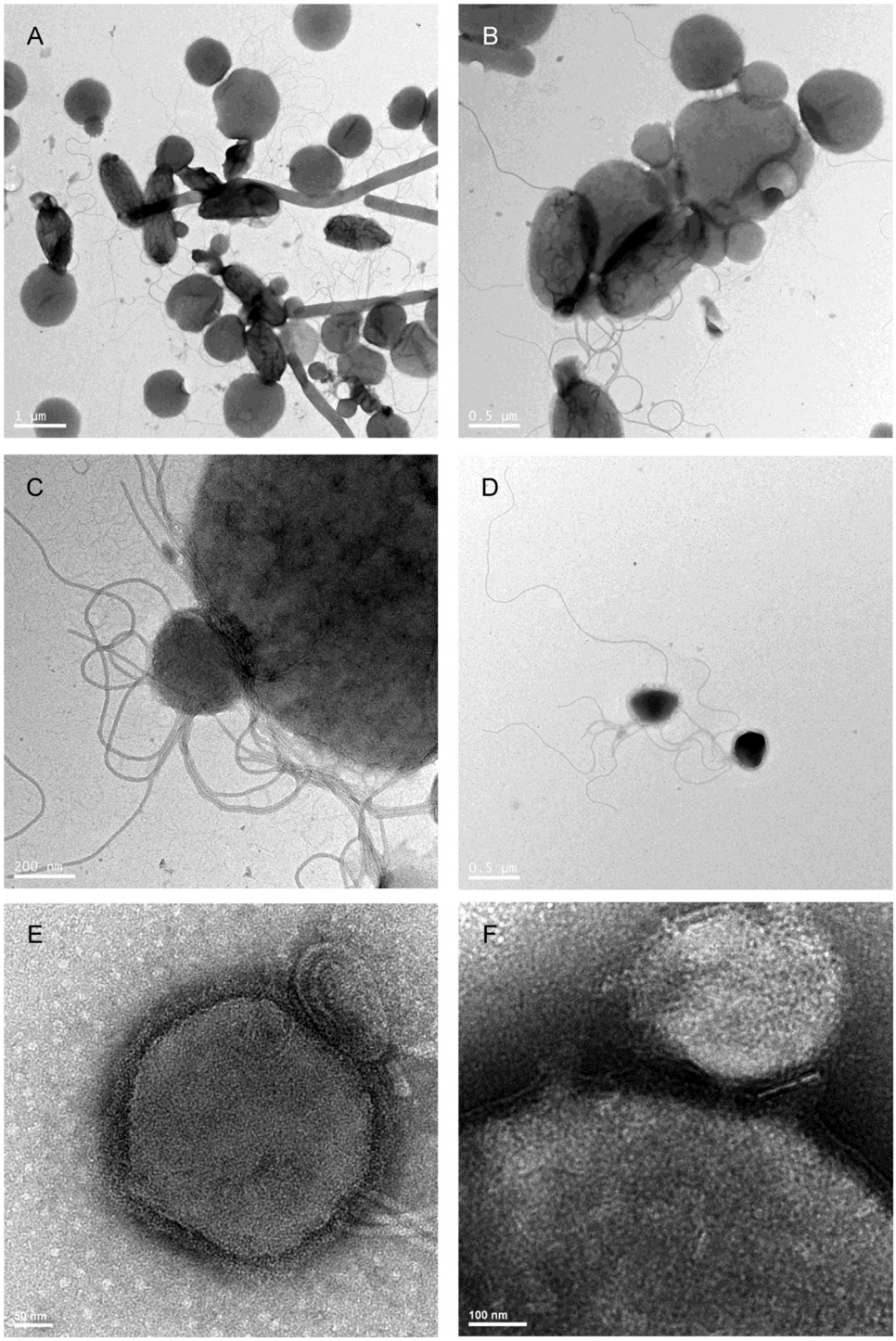

Fig. 1. Negatively stained transmission electron micrographs (TEM) of the New Zealand enrichment culture. (A) Enrichment culture with nanoarchaeotes, (B) several nanoarchaeotes attached to host coccus, (C) nanoarchaeote attached to host with lophotrichous archaeal flagella, (D) unattached nanoarchaeotes with lophotrichous flagella, (E) detailed view of archaeal flagella attachment, and (F) cell connection between host and nanoarchaeote.

defined enrichments were reconstructed by combining several of the isolate cultures described above. Cultures were grown for four days and then inoculated with nanoarchaeotes by adding 0.22 μm-filtered spent nanoarchaeote-containing media (10% v/v). These mixed cultures were then monitored by qPCR after four, five and six days of incubation. Very low nanoarchaeotal numbers were detected in one reconstructed culture after six days of incubation. This culture was then reinoculated several times into fresh media until a stable culture was established. The resulting mixed culture had $2–5 \times 10^7$ nanoarchaeotes/mL. All further isolation attempts and metagenomic analyses of the nanoarchaeote-host system were conducted using this reconstructed culture.

Isolation of the nanoarchaeote in co-culture and the host in pure culture

Subsequently, we successfully isolated the New Zealand nanoarchaeote and its crenarchaeotal host in co-culture by screening

several 10^{-3} dilutions. The purity of the co-cultures was confirmed by PCR. On the basis of 16S rRNA gene sequence, the nanoarchaeote (strain Ncl-1) was most closely related (96.6%) to a clone 16S rRNA gene sequence (Genbank EF562624.1 [14]) from Tokaanu hot springs in New Zealand and only 88.4% and 84.4% similar to *Cand.* Nps. acidilobi and *N. equitans*, respectively. Phylogenetic analysis of the 16S rRNA gene also confirmed the position of Ncl-1 in the *Nanoarchaeota* (Fig. S1).

The New Zealand host (strain NZ3^T) was also isolated after several rounds of dilution-to-extinction transfers. The purity of the isolate was verified by amplification and sequencing of the 16S rRNA gene, which was found to contain four introns (2273 bp total gene length; Fig. 2). Introns in archaeal 16S rRNA genes have been widely reported in the *Crenarchaeota*, particularly in the *Desulfurococcales* and *Thermoproteales* [13,38,40,57,75,102], and often encode homing endonucleases which may facilitate their spread throughout microbial communities [1,40,57]. The largest of the four predicted introns in the NZ3^T 16S rRNA gene has extensive secondary structure and contains a predicted homing endonuclease, while the other three introns form small hairpin structures [111]. After trimming the introns from the sequence, the NZ3^T 16S rRNA gene showed highest sequence similarity (~93%) to clones from deep-sea hydrothermal vents (Genbank DQ228585.1, DQ228604.1, unpublished) and 92% to *Ignisphaera aggregans* [73]. Based on maximum likelihood phylogenetic analysis of the 16S rRNA gene, strain NZ3^T formed a deeply-branching clade in the *Desulfurococcaceae* with *I. aggregans* (Fig. S2).

Characterization of the host, strain NZ3^T

The morphology of strain NZ3^T was analyzed using phase contrast microscopy and TEM. Cells were coccoid, approximately 1 μm in diameter, and no archaeal flagella were observed. NZ3^T grew between 60 and 90 °C, growing best between 82–83 °C (Table S3). No growth was observed at 55 or 95 °C. The isolate grew from pH 4.9 and 7.2, with optimal growth at pH 6.0, but no growth observed at pH 4.6 or 7.2.

Strain NZ3^T grew well on peptide-rich substrates (0.1% w/v), including yeast extract, tryptone, casamino acids and gelatin. However, no growth was observed on sugars, fatty acids, or alcohols, including fructose, glucose, mannose, ribose, sucrose, xylose, acetate, butyrate, propionate, sorbitol, glycerol, cellulose or starch. Thiosulfate (2.5–10 mM) was required as a terminal electron acceptor and could not be replaced by 2.5 or 10 mM L-cysteine-HCl, sulfite, nitrate or elemental sulfur.

Strain NZ3^T grew in the presence of 0–0.5% NaCl (w/v), but no growth was observed at 1% NaCl. NZ3^T is an obligate anaerobe, as trace amounts of oxygen in the headspace (3% v/v) inhibited growth.

The membrane lipids of strain NZ3^T consisted of glycerol dialkyl glycerol tetraethers, with zero to four cyclopentane moieties with relative proportions of 39%, 18%, 22%, 13% and 8% respectively (Figs. S3 and S4). Although no lipid composition data is available for *I. aggregans*, the five core lipids found in NZ3^T were also identified in *Thermosphaera aggregans*, another member of the *Desulfurococcaceae* [36,94]. Intact polar lipid analysis of the polar head groups revealed the presence of monohexose, dihexose, trihexose, phosphoinositol and hexose phosphoinositol (Fig. S5, Table S4).

The genome of strain Ncl-1 more closely resembles its terrestrial relatives than marine N. equitans

In order to gain insight into the relationship between the nanoarchaeote and its host, their draft genomes were obtained from single-cell sorted metagenomes of the enrichment. Using the 16S rRNA genes, the nanoarchaeote and host bins were identified. The nanoarchaeote strain Ncl-1 draft genome consists of nine contigs, totaling 0.576 Mbp, with a 32% G + C content (Table S5). Using CheckM, the draft genome contains 121 of the 149 marker genes used to estimate completeness, while the closed genomes of *N. equitans* and *Cand.* Nps. acidilobi have 118 and 119 of the marker genes, respectively. Ncl-1 is 52% similar to *Cand.* Nps. acidilobi and 43% similar to *N. equitans* based on average amino acid identity (AAI) [90], indicating genus-level divergence [47]. Concatenated ribosomal protein phylogeny also supports the position of Ncl-1 in the *Nanoarchaeota* (Fig. 3).

On a genomic level, Ncl-1 most closely resembles its terrestrial relatives, "Nanobsidianus stetteri" Nst1 and *Cand.* Nps. acidilobi (Table S5), and like other nanoarchaeotes, it lacks the ability to synthesize most metabolic precursors such as nucleotides, amino acids, cofactors and lipids. Ncl-1 shows modest synteny with the genomes of *Cand.* Nps. acidilobi and *N. equitans* (Fig. S6A, B), and it is also highly syntenic with a nanoarchaeote draft genome obtained from a New Zealand hot spring enrichment metagenome collected two years prior, in 2013 [43] (Fig. S6C). As "N. stetteri" Nst1 and *Cand.* Nps. acidilobi are very similar at the genomic level, the majority of the comparative analyses in this study were done with the closed genome of *Cand.* Nps. acidilobi. Like *Cand.* Nps. acidilobi, the Ncl-1 draft genome is about 100 kb larger than *N. equitans*, has partial glycolytic and gluconeogenic pathways, lacks an apparent ATP synthase complex and contains a full suite of archaeal flagellar genes [106].

In contrast to *N. equitans*, which only has one of the enzymes in the Embden–Meyerhof–Parnas (EMP) glycolysis pathway [10], Ncl-1 encodes several of the EMP pathway genes. Like *Cand.* Nps. acidilobi, however, glucokinase, phosphofructokinase (PFK) and fructose 1,6-bisphosphate aldolase (FBPA) were not detected, suggesting the EMP pathway may not be functional in terrestrial nanoarchaeotes. The non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) and pyruvate kinase (PK) genes found in *Cand.* Nps. acidilobi were not found in the Ncl-1 draft genome, although the PK gene also was not expressed in the proteome of *Cand.* Nps. acidilobi [106]. As an alternative, the PK step may be catalyzed in both *Cand.* Nps. acidilobi and Ncl-1 by phosphoenolpyruvate synthase (PEPS, Ncl-1 451, 487), as reported in *Thermococcus kodakarensis* [10,37,106]. Nonetheless, the EMP glycolytic pathway is likely nonfunctional in either *Cand.* Nps. acidilobi or Ncl-1.

Most of the genes for gluconeogenesis, several which are bidirectional, are present in both terrestrial nanoarchaeotes (absent in *N. equitans*) and are expressed in the proteome of *Cand.* Nps. acidilobi [106]. It is therefore likely that gluconeogenesis can proceed to some extent in these two nanoarchaeotes and its primary role may be to synthesize activated sugars for protein and lipid glycosylation, as *Cand.* Nps. acidilobi has a glycosylated, lectin-reactive surface [106]. Although the glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase (GAPDH/PGK) couple was not detected in either *Cand.* Nps. acidilobi or Ncl-1, Wurch et al. [106] proposed that its function may be replaced by a reversible ferredoxin-dependent aldehyde oxidoreductase (Ncl-1 615). Furthermore, while both terrestrial nanoarchaeotes have an acetyl-CoA synthase (Ncl-1 481, 606), it was not expressed in the *Cand.* Nps. acidilobi proteome under tested culture conditions [106], leaving it unclear whether *Cand.* Nps. acidilobi and Ncl-1 can convert acetate to acetyl-CoA for gluconeogenesis, or if acetyl-CoA is obtained from their hosts or other sources.

Strain Ncl-1 has two ABC-type multidrug transporter subunits (Ncl-1 256–257), two ABC-type antimicrobial peptide transporter subunits (Ncl-1 473, 476), and two gene copies of a cation transport ATPase (Ncl-1 290, 573) which all show strong homology to transporters reported for *Cand.* Nps. acidilobi. However, the major

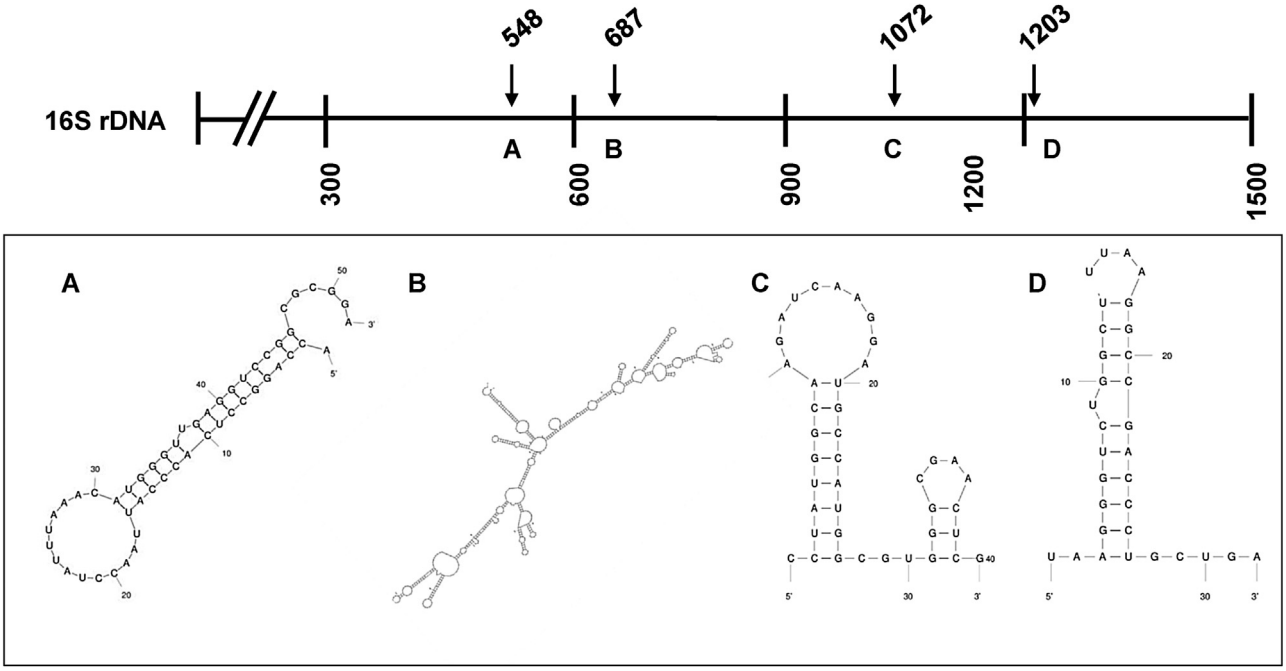

Fig. 2. Predicted secondary structure and placement of intervening sequences in the 16S rRNA gene of strain NZ3^T. Structures were predicted and drawn in Mfold. Only the most energetically favorable conformations are presented.

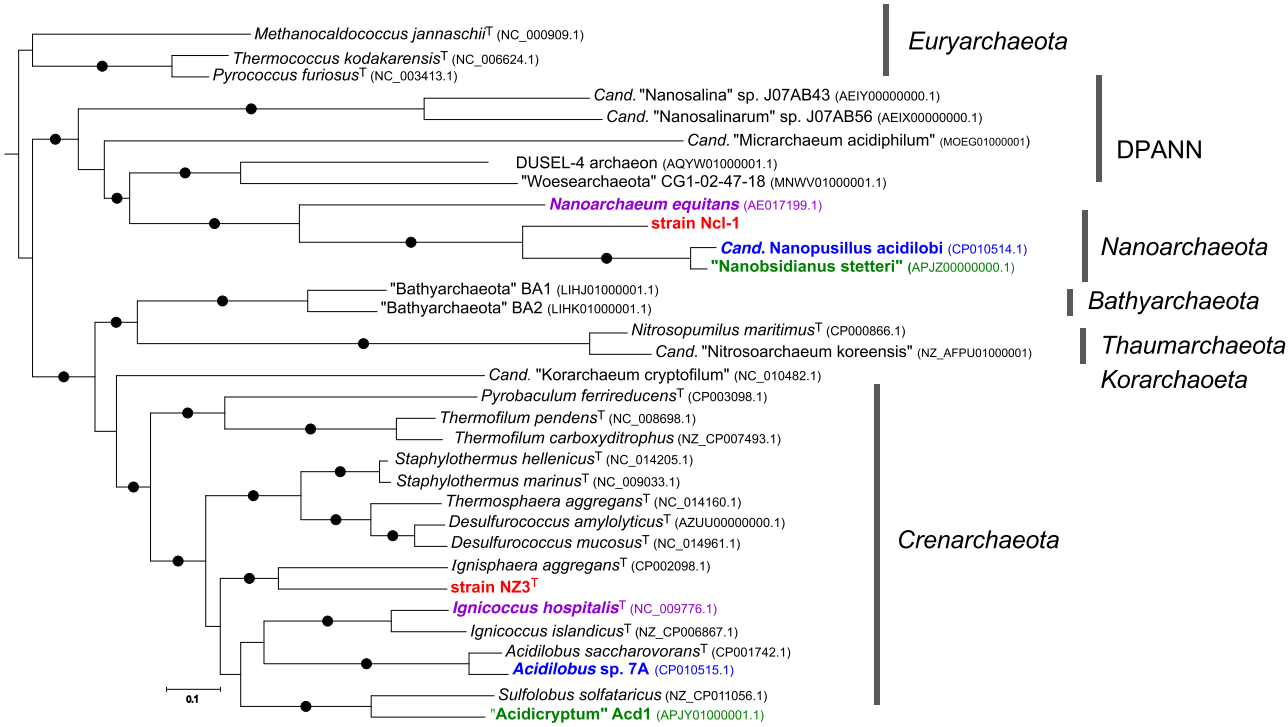

Fig. 3. Phylogenetic analysis of 16 concatenated ribosomal proteins (rpL2, 3, 4, 5, 6, 14, 15,16, 18, 22, 24 and rpS3, 8, 10, 17, 19) using RAxML. The tree was rooted in the *Euryarchaeota*. Closed circles represent bootstrap support of ≥70%. The four different *Nanoarchaeota*-host systems are in red, blue, purple and green. The scale bar represents 0.1 amino acid substitutions per site.

facilitator superfamily permeases were not detected in the draft genome. In contrast, Ncl-1 has two Trk-system proteins involved in potassium uptake (Ncl-1 249–250) that have not previously been reported in the *Nanoarchaeota*.

Strain Ncl-1 and *Cand.* Nps. acidilobi encode for archaeal flagella using very similar gene arrangements (Fig. S7A and B), both containing the essential *fla* genes required for assembly and function [2], but lacking the traditional operon structure found in other *Archaea* (Fig. S7C). Like *Cand.* Nps. acidilobi, Ncl-1 has a *flaD/E* gene (Ncl-1 218) typical of *Euryarchaeota* [2]. TEM micrographs revealed that archaeal flagella genes are functional in strain Ncl-1 and expressed under cultivation conditions (Fig. 1C–E). Strain Ncl-1 is often seen with several (~5) lophotrichous archaeal flagella, both when associated (Fig. 1C) and detached from its host (Fig. 1D–E). If

motile, the nanoarchaeotal flagella may play a role in host-seeking behavior, as proposed for marine obligate intracellular parasitic *Chlamydiae* [17], or they may allow the nanoarchaeote to detach and search for a new host [106]. Alternatively, the nanoarchaeotal flagella may mediate adhesion to surfaces [39,110] or attachments between the symbiont and its host [93]. However, the presence of archaeal flagella presents a conundrum for both Ncl-1 and *Cand.* Nps. acidilobi, as neither possesses any apparent means of producing ATP to fuel motility.

Strain Ncl-1 shares additional similarities with the central metabolic genes of *N. equitans* and *Cand.* Nps. acidilobi. Like all characterized nanoarchaeotes, Ncl-1 has a non-discriminating Glu-tRNA synthetase (Ncl-1 520) and both of the amidotransferase subunits required for transamidation of Glu-tRNA^{Gln} to Gln-tRNA^{Gln} (Ncl-1 159–160). Both *Cand.* Nps. acidilobi and Ncl-1 appear to synthesize Asn-tRNA^{Asn} directly via an asparaginyl-tRNA synthetase (Ncl-1 446), instead of using the transamidation reaction found in *N. equitans*. However, Ncl-1 encodes a class I lysyl-tRNA synthetase (Ncl-1 78) similar to marine *N. equitans*, thought to be the ancestral gene form [83], while *Cand.* Nps. acidilobi harbors the class II enzyme. Similar to *Cand.* Nps. acidilobi, Ncl-1 can also convert L-cysteine to L-alanine via a cysteine desulfurase (Ncl-1 421). Furthermore, Ncl-1 possesses some unique nucleotide biotransformation capabilities not found in either *Cand.* Nps. acidilobi or *N. equitans*, including a purine-nucleoside phosphorylase (Ncl-1 611), a CTP synthase (Ncl-1 590), and a nucleoside-triphosphatase THEP1 (Ncl-1 633).

The Ncl-1 draft genome contains 41 tRNAs; encoding for all 20 standard amino acids. Like all described nanoarchaeotes, Ncl-1 contains several *cis*-spliced tRNAs which are common throughout the *Archaea* [27,45,101,105,106]. However, the *trans*-spliced tRNAs identified in *N. equitans* [85,86] were not found in either Ncl-1 or *Cand.* Nps. acidilobi. Similarly, both the genomes of *Cand.* Nps. acidilobi and Ncl-1 encode for RNase P (inferred in Ncl-1 on the basis of RNase P protein subunits) while it was not identified in the genome of *N. equitans* [55,87]. The Ncl-1 draft genome has a common set of split protein coding genes, shared by all described nanoarchaeotes (Table 1). This provides further evidence that genome reduction in the *Nanoarchaeota* resulted in stochastic fragmentation, with genes often split in the same location [83]. Some unique split genes also occur across the nanoarchaeote genomes, which is not surprising given the phylogenetic diversity of the three cultivated nanoarchaeotes.

Similar to its marine relative *N. equitans*, Ncl-1 has a putative CRISPR-Cas system, representing the first described CRISPR-Cas system for a terrestrial nanoarchaeote [68,83,106]. The system contains 43 spacer regions and a suite of Type 1 *cas* genes. Although several of the *cas* genes show strong homology to their counterparts in *N. equitans*, the gene arrangement is quite different (Fig. S8) and the system contains an additional *cas6* gene (Ncl-1 284) not identified in *N. equitans*. The target of the Ncl-1 CRISPR-Cas system is unclear as the spacer regions do not show significant homology (e-value $\leq 10^{-5}$) to viral sequences in the NCBI nucleotide database. Although no CRISPR-Cas system has been previously reported in terrestrial nanoarchaeotes, a nanoarchaeotal draft genome from Nymph Lake, YNP, showed evidence of integrated prophage DNA [68], demonstrating that geographically distant nanoarchaeotes are vulnerable to viral infection.

Metabolic insights from the draft genome of the host, NZ3^T

To explore the metabolic capabilities of NZ3^T further, the draft genome was reconstructed from low-diversity metagenomic data as previously described. The NZ3^T draft genome consists of 19 contigs, with a 41.9% G+C content and 97.5% completion based on CheckM (Table S5). Phylogenetic reconstruction using a set of 16 ribosomal proteins (Fig. 3) confirmed the placement of NZ3^T in the *Desulfurococcaceae*, in a deeply-branching clade with *I. aggregans*. The genome is 45% similar to *I. aggregans* based on AAI, which is consistent with the level of 16S rRNA gene sequence divergence between the two organisms [47]. The strain NZ3^T genome also shows modest levels of synteny to *I. aggregans* (Fig. S6D), and the two strains are similar in size (~1.808 Mbp and 1.876 Mbp, respectively) [29]. Forty-nine tRNA genes (19 of 20 standard amino acids), three rRNA genes and RNase P were also identified in the NZ3^T genome. Similar to *Fervidicoccus fontis*, another member of the *Thermoprotei*, NZ3^T appears to be auxotrophic for a variety of amino acids and coenzymes, such as biotin and coenzyme A [51].

Consistent with its growth on peptide-rich substrates, the NZ3^T draft genome contains secreted and cytosolic peptidases and proteases, and a suite of transporters to import dipeptides, oligopeptides and amino acids into the cell. Once inside the cell, amino acid degradation likely proceeds using pyruvate:ferredoxin oxidoreductases (NZ3 1006–1009; 1927–1930) and indolepyruvate:ferredoxin oxidoreductases (NZ3 279–280; 1116–1117) as described for members of the *Thermococcaceae* [33,58,61]. Breakdown of amino acids provides NZ3^T with limited ATP and a pool of reduced ferredoxin molecules.

The NZ3^T genome has incomplete gluconeogenesis and EMP glycolysis pathways, and the Entner–Doudoroff glycolysis pathway and pentose phosphate pathway were not detected. Possibly, NZ3^T may utilize an archaeal type III RuBisCO (NZ3 708, 1082) to generate 3-phosphoglycerate (3-PGA) as proposed by Sato et al. [91]. 3-PGA could then be converted to acetate using enzymes involved in glycolysis and amino acid fermentation, generating ATP for the cell.

Despite its dependence on thiosulfate as a terminal electron acceptor, no thiosulfate reductase gene was identified in the NZ3^T draft genome. However, it is possible that the gene may be encoded in one of the unsequenced portions of the genome. Alternately, NZ3^T may utilize a divergent gene for thiosulfate reduction that has yet to be identified.

Strain NZ3^T has several different hydrogenases, including two membrane-bound [NiFe] group 4b hydrogenase clusters which evolve molecular hydrogen [31]. One of these clusters contains a putative catalytic carbon monoxide dehydrogenase subunit *cooS* (NZ3 969), suggesting that NZ3^T may be able to conserve energy through carboxydotrophic hydrogenogenesis (coupling CO oxidation and proton reduction) in a similar manner to that of *Thermococcus onnurineus* and *Thermococcus* sp. AM4 [52,96,97]. The NZ3^T *cooS* gene shows strong phylogenetic relatedness to *cooS* genes found in known carboxydotrophs such as *Thermococcus barophilus* (Genbank NC_014804.1 [104]) and *Thermococcus* sp. AM4 (Genbank CP002952.1 [76]) (Fig. S9). However, additional physiological studies will be required to determine whether or not CO oxidation is functional in strain NZ3^T. The second [NiFe] group 4b hydrogenase cluster (NZ3 314–329) in the draft genome encodes a multi-subunit Na^+/H^+ antiporter which may facilitate the formation of a sodium ion gradient across the membrane [31]. This sodium gradient may be used to generate ATP using an archaeal-type ATP synthase (NZ3 1842–1845, 1847–1849), as reported for *Desulfurococcus kamchatkensis*, *Pyrococcus furiosus*, and *Methanosarcina acetivorans* [82,88,92]. The NZ3^T genome also encodes a [NiFe] group 3A hydrogenase cluster containing a coenzyme F_{420}-reducing hydrogenase (NZ3 1861) similar to those found in methanogens and *T. onnurineus* [31,41,52,53]. Phylogenetic analysis clusters the NZ3^T gene with coenzyme F_{420}-reducing hydrogenases from *T. onnurineus* and other non-methanogenic lineages with high confidence (Fig. S10). To our knowledge, this represents the first example of a coenzyme F_{420}-reducing hydrogenase reported in the *Crenarchaeota*. As suggested for *T. onnurineus*,

Table 1
Comparison of genomic features in strain Ncl-1 and other *Nanoarchaeota*.[a]

Characteristic	Strain Ncl-1	*N. equitans*	"*N. stetteri*" Nst1	*Cand.* Nps. acidilobi
Split protein coding genes				
Reverse gyrase	Ncl-1 419-492	NEQ 318-434	Nst 337-402	Nps 2030-2705
Glu-tRNA^{Gln} amidotransferase subunit E	***Ncl-1 160***	NEQ 245-396	Nst 197-449	Nps 2280-3415
Predicted RNA-binding protein	Ncl-1 37-631	NEQ 438-506	Nst 176-251	Nps 30-3140
Archaeosine tRNA-guanine transglycosylase	Ncl-1 339-649	NEQ 124-305	Nst 096-232	Nps 1135-3235
RNA polymerase subunit B	***Ncl-1 121***	NEQ 156-173	Nst 632-633	Nps 545-550
Large helicase-related protein	Ncl-1 158-643	NEQ 003-409	Nst 172-239	Nps 745-3195
DNA polymerase I	Ncl-1 402	NEQ 068-528	Nst 417	Nps 2105
Topoisomerase I	Ncl-1 39	NEQ 045-324	Nst 174	Nps 45
P-loop ATPase acetyltransferase fusion protein	Ncl-1 401	NEQ 096-495	Nst 401	Nps 2025
Alanyl-tRNA synthetase	Ncl-1 77	NEQ 211-547	Nst 054	Nps 290
Diphthamide synthase sub. DPH2	Ncl-1 5-382	Absent	Nst 222-440	Nps 3285-2235
Uncharacterized conserved protein (arCOG 04253)	Ncl-1 582	Absent	Nst 474-80	Nps 2410
Glucosamine fructose-6-phosphate aminotransferase	***Ncl-1 169-171***	Absent	Absent	Absent
cis-Spliced tRNAs	Ile Tyr, Met, ***Leu, Asp***	Ile, Tyr, Met, Trp	Ile, Tyr	Ile, Tyr
trans-Spliced tRNAs	None	iMet, His, Lys, Gln, Glu (2)	None	None
RNase P	Present	Absent	Present	Present
Gluconeogenesis/glycolysis	Present (partial)	Absent	Present (partial)	Present (partial)
Polyamine biosynthesis	Present	Absent	Present	Present
ATP synthase complex	Absent	Incomplete	Absent	Absent
Glutamate dehydrogenase	Present	Present	Absent	Absent

Bold-italics depicts where the new Ncl-1 differs from other described Nanoarchaeota.
[a] Table modified from Podar et al. [83].

this hydrogenase likely does not utilize coenzyme F_{420}, since there is no genomic evidence that either organism is capable of synthesizing the coenzyme [41,53]. While its function in non-methanogens remains unclear, transcriptomic data from *T. onnurineus* indicates that the coenzyme F_{420}-reducing hydrogenase impacts the expression of CO oxidation genes, potentially pointing to a larger role in central metabolism [53].

Genomic evidence suggests that NZ3^T encodes for synthesis of an S-layer (NZ3 717), similar to *Acidilobus* sp. 7A, the host of terrestrial *Cand.* Nps. acidilobi [106]. This is in contrast with *I. hospitalis*, the host of *N. equitans*, which has a distinctive double membrane system and lacks an S-layer [78,84]. Based on a recent study using electron tomography, the double membrane structure in *I. hospitalis* appears to play a crucial role in its association with *N. equitans*, as the host and symbiont form a cytoplasmic bridge at their attachment site [84]. A similar pore-like structure was detected between NZ3^T and its nanoarchaeotal symbiont at their attachment site, with no membrane clearly visible between the cells (Fig. 1F) and suggests an equally intimate association between the terrestrial nanoarchaeote and its host. However, given the extreme differences in host outer membrane structure, it is likely that terrestrial nanoarchaeotes have evolved a unique mechanism of recognizing, attaching, and interacting with their hosts.

Proposal of a novel Candidatus *taxon in the* Nanoarchaeota *and a novel genus and species in the* Desulfurococcaceae

The New Zealand nanoarchaeote strain Ncl-1 is distinct from its described relatives at both a phylogenetic and a genomic level. While 16S rRNA gene and concatenated ribosomal protein phylogenetic trees place Ncl-1 in the *Nanoarchaeota*, the 16S rRNA gene of the strain is divergent from its described relatives (~84–88% gene sequence similarity). AAI analysis also suggests genus-level divergence between Ncl-1 and the cultivated taxa *Cand.* Nps. acidilobi and *N. equitans* (45–52%). Although the genome of Ncl-1 shows stronger homology to its terrestrial relatives, it contains several genes not previously found in the *Nanoarchaeota*. Ncl-1 is also the first cultivated *Nanoarchaeota* from a New Zealand geothermal spring, and its host is a new crenarchaeotal genus. The clear phylogenetic divergence between Ncl-1 and other described nanoarchaeotes suggests that it represents a novel genus and species in the *Nanoarchaeota*. The formal proposal of a new *Candidatus* taxon in the *Nanoarchaeota*, *Candidatus* Nanoclepta minutus, is given in Table 2, with the Taxonumber CA00029.

Strain NZ3^T forms a clade in the *Desulfurococcaceae* with *I. aggregans*. However, NZ3^T is only ~92% similar to *I. aggregans* by 16S rRNA gene similarity and 45% similar by AAI. Unlike *I. aggregans*, NZ3^T requires thiosulfate as a terminal electron acceptor and grows well on several proteinaceous substrates but cannot use any sugars for growth. Moreover, NZ3^T grows optimally between 82–83 °C, which is much lower than the optimal temperature (92–95 °C) of *I. aggregans*. Given these physiological and phylogenetic differences, we propose that strain NZ3^T represents a new species of a novel genus in the *Desulfurococcaceae*. The formal proposal of the novel genus *Zestosphaera* gen. nov. and the species *Zestosphaera tikiterensis* sp. nov. is provided in Table 2, with the Taxonumbers GA00064 and TA00512, respectively.

Conclusions

Here we expand on nanoarchaeote-host symbiotic systems by describing a fourth association. A stable co-culture containing a novel nanoarchaeote and its host, a new genus in the *Desulfurococcaceae*, was cultivated from a geothermal hot spring in New Zealand. The New Zealand nanoarchaeote closely resembles other terrestrial nanoarchaeotes, with the presence of archaeal flagella and no apparent ATP synthase genes, but it also contains a CRISPR-Cas system like its marine relative *N. equitans*. The fragmentation of archaeal flagellar genes and the presence of shared split protein-coding genes distributed among marine and terrestrial nanoarchaeotes provide further evidence that genome reduction and fragmentation in this lineage began before the marine and terrestrial taxa diverged. The mechanisms by which phylogenetically different hosts interact with their nanoarchaeotal symbionts remain elusive. However, as more nanoarchaeote-host systems are characterized, additional insights will emerge about the cell-cell interactions in these symbioses, and whether host metabolism and ultrastructure have played roles in the evolutionary history of the symbiosis.

The New Zealand *Nanoarchaeota*-host system also points to the challenges associated with describing a cultivated organism that is only viable in the presence of its host. Under the current system

Table 2
Descriptions of *Candidatus* Nanoclepta minutus, *Zestosphaera* gen. nov. and *Zestosphaera tikiterensis* sp. nov., based on the digital protologues CA00029, GA00064 and TA00512, respectively. Protologues are available at http://imedea.uib-csic.es/dprotologue/.

Characteristic	*Candidatus* Nanoclepta gen. nov.	*Candidatus* Nanoclepta minutus sp. nov.	*Zestosphaera* gen. nov.	*Zestosphaera tikiterensis* sp. nov.
Taxonumber	CA00029	CA00029	GA00064	TA00512
Genus name	*Candidatus* Nanoclepta		*Zestosphaera*	
Genus status	gen. nov.		gen. nov.	
Species name		*Candidatus* Nanoclepta minutus		*Zestosphaera tikiterensis*
Specific epithet		minutus		*tikiterensis*
Species status		sp. nov.		sp. nov.
Etymology	Na.no.clep'ta. Gr. masc. n. *nanos,* a dwarf; Gr. masc. n. *kleptes,* a thief; N.L. masc. n. *Nanoclepta,* a small thief, a small organism that steals from its host	mi.nu'tus. L. masc. adj. *minutus,* small	Zes.to.sphae'ra. Gr. adj. *zestos,* hot, boiling; Gr. fem. n. *sphaera,* a ball, a sphere; N.L. fem. n. *Zestosphaera,* a hot sphere	ti.ke.ter.en'sis. N.L. fem. adj. *tikiterensis,* referring to Tikitere
Has the taxon been subjected to emendation?	No	No	No	No
Number of stains in study	1	1	1	1
Type species of the genus			*Zestosphaera tikiterensis* NZ3^T	
Designation of the type strain				*Zestosphaera tikiterensis* NZ3^T
Taxonumber of the type species			TA00512	
Title	A New Symbiotic Nanoarchaeote (Candidatus Nanoclepta minutus) and its host (*Zestosphaera tikiterensis* gen. nov., sp. nov.) from a New Zealand hot spring			
Authors	St. John E, Liu Y, Podar M, Stott M, Meneghin J, Chen Z, Lagutin K, Mitchell K, Reysenbach AL			
Corresponding author	Anna-Louise Reysenbach			
Email of the corresponding author	reysenbacha@pdx.edu			
Submitter	Emily St. John			
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Country of origin	New Zealand	New Zealand	New Zealand	New Zealand
Region of origin	Tikitere	Tikitere	Tikitere	Tikitere
Geographic location	Hell's Gate, Tikitere, NZ	Hell's Gate, Tikitere, NZ	Hell's Gate, Tikitere, NZ	Hell's Gate, Tikitere, NZ
Latitude	38.0654°S	38.0654°S	38.0654°S	38.0654°S
Longitude	176.3595°E	176.3595°E	176.3595°E	176.3595°E
Sampling date	April 16, 2015	April 16, 2015	April 16, 2015	April 16, 2015
Source of isolation	Water and sediment	Water and sediment	Water and sediment	Water and sediment
Temperature of the sample (°C)	78.5	78.5	78.5	78.5
pH of the sample	6.48	6.48	6.48	6.48
Strain collection numbers			OCM 1214; DSM 107634	OCM 1214; DSM 107634
GC mol%	32.2	32.2	41.9	41.9
16S rRNA accession number	MH298645	MH298645	MH252993	MH252993
Designation of the type MAG	*Candidatus* Nanoclepta minutus	*Candidatus* Nanoclepta minutus		
Genome accession number	MWMI00000000	MWMI00000000	NBVN00000000	NBVN00000000
Genome status	Draft	Draft	Draft	Draft
Genome size (kbp)	576	576	1808	1808
Growth medium, incubation conditions (temperature, pH, and further information) used for standard cultivation	Media is a modified DSMZ media #88 with (g/L): $Na_2O_3S_2{\cdot}5H_2O$, 2.0; NaOH, 0.16; yeast extract, 1.0; tryptone, 1.0; casein hydrolysate, 0.5; L-cysteine-HCl, 0.0625; resazurin, 0.001; $FeCl_3{\cdot}6H_2O$, 0.004; $MnCl_2{\cdot}4H_2O$, 0.00036; glycerol, 10.0 mL/L, and 10 mL DSMZ #141 trace mineral solution. pH is adjusted to 6.0–6.1 and N_2/CO_2 (80–20, v/v) is in the headspace. Isolation was carried out at 80–85 °C.			
Conditions of preservation	Liquid cultures are mixed with 10% glycerol (v/v) and frozen in liquid nitrogen.			
Gram stain			Negative	Negative
Cell shape			Coccus	Coccus
Cell size (length or diameter)			~1 μm	~1 μm
Motility			Nonmotile	Nonmotile
Temperature range (°C)			60–90	60–90

Temperature optimum (°C)			82–83	82–83
pH range			4.9–7.0	4.9–7.0
pH optimum			6.0	6.0
pH category			Neutrophile	Neutrophile
Range of NaCl concentration for growth			0–0.5	0–0.5
Relationship to O_2	Anaerobe	Anaerobe	Anaerobe	Anaerobe
O_2 conditions for strain testing			Anaerobiosis	Anaerobiosis
Carbon source used (class of compounds)			Amino acids, proteins	Amino acids, proteins
Carbon source used (specific compounds)			Casamino acids, tryptone, yeast extract, gelatin	Casamino acids, tryptone, yeast extract, gelatin
Carbon sources not used (specific compounds)			Acetate, butyrate, cellulose, fructose, glucose, glycerol, mannose, propionate, ribose, sorbitol, starch, sucrose, xylose	Acetate, butyrate, cellulose, fructose, glucose, glycerol, mannose, propionate, ribose, sorbitol, starch, sucrose, xylose
Terminal electron acceptor			Thiosulfate	Thiosulfate
Habitat	Hot spring	Hot spring	Hot spring	Hot spring
Biotic relationship	Symbiotic	Symbiotic	Free-living	Free-living
Relationship to host		Symbiotic with *Zestosphaera tikiterensis*		
DNA extraction method	DNeasy Blood and Tissue Kit (Qiagen)			
Sequencing technology	Illumina MiSeq			
Assembly	1			
Assembly software used	IDBA-UD v.1.1.0, SPAdes genome assembler v.3.9.1-Linux			
Binning software used	MaxBin v.1.4.5, Emergent self-organizing mapping (ESOM)			
Miscellaneous extraordinary features relevant for the description		Obligate ectosymbiont of *Zestosphaera tikiterensis.* Best growth from 80–85 °C, but growth observed from 60–90 °C. Archaeal flagella present. Does not contain ATP synthase genes.		This isolate is the host organism of the ectosymbiotic *Cand.* Nanoclepta minutus, but it can grow without its symbiont. Requires thiosulfate for growth.

of nomenclature, regulated by the International Code of Nomenclature of *Bacteria*, a pure culture is required for the valid naming and recognition of a novel taxon [46,50]. However, no provision is made for obligate symbionts that are cultivated in the minimum consortium needed for growth (co-culture). For some intracellular pathogens, such as *Lawsonia* [64], *Diplorickettsia* [66], and members of the *Chlamydiae* [28,30], valid names may be assigned if the strain is cultivable in cell culture. However, this option is not available for many environmental taxa requiring a specific host for cultivation, such as ectosymbiotic *Nanoarchaeota*, or the diverse epibiotic *Archaea* and *Bacteria* that colonize ciliates [12,23,95]. While the *Candidatus* designation provides a means of identifying these novel lineages, it is a provisional status and implies that the organism will be isolated in the future, which is not feasible for obligate symbionts, parasites, or some epibionts [46]. Ultra-small genomes recovered from the archaeal DPANN superphylum [6,89] and bacterial Candidate Phyla Radiation [18] suggest a large diversity of yet-uncultivated microbial taxa that may rely on hosts for survival. Looking ahead, as more of these lineages are discovered and cultivated, the coherent classification of symbionts will require a greater acceptance and standardization of the *Candidatus* designation, or a novel system of symbiont nomenclature. Further complicating matters are names that have become adopted as validly described through repeated use such as *N. equitans*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.08.005.

References

[1] Aagaard, C., Dalgaard, J.Z., Garrett, R.A. (1995) Intercellular mobility and homing of an archaeal rDNA intron confers a selective advantage over intron-cells of *Sulfolobus acidocaldarius*. Proc. Natl. Acad. Sci. U. S. A. 92 (26), 12285–12289.
[2] Albers, S.-V., Jarrell, K.F. (2015) The archaellum: how *Archaea* swim. Front. Microbiol. 6, 23, http://dx.doi.org/10.3389/fmicb.2015.00023.
[3] Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410.
[4] Anantharaman, K., Brown, C.T., Hug, L.A., Sharon, I., Castelle, C.J., Probst, A.J., Thomas, B.C., Singh, A., Wilkins, M.J., Karaoz, U., Brodie, E.L., Williams, K.H., Hubbard, S.S., Banfield, J.F. (2016) Thousands of microbial genomes shed light on interconnected biogeochemical processes in an aquifer system. Nat. Commun. 7, 13219, http://dx.doi.org/10.1038/ncomms13219.
[5] Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O. (2008) The RAST server: rapid annotations using subsystems technology. BMC Genom 9, 75, http://dx.doi.org/10.1186/1471-2164-9-75.
[6] Baker, B.J., Comolli, L.R., Dick, G.J., Hauser, L.J., Hyatt, D., Dill, B.D., Land, M.L., Verberkmoes, N.C., Hettich, R.L., Banfield, J.F. (2010) Enigmatic, ultrasmall, uncultivated *Archaea*. Proc. Natl. Acad. Sci. U. S. A. 107 (19), 8806–8811.
[7] Baker, B.J., Saw, J.H., Lind, A.E., Lazar, C.S., Hinrichs, K.-U., Teske, A.P., Ettema, T.J.G. (2016) Genomic inference of the metabolism of cosmopolitan subsurface *Archaea*, *Hadesarchaea*. Nat. Microbiol. 1, 16002, http://dx.doi.org/10.1038/nmicrobiol.2016.2.
[8] Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19 (5), 455–477.
[9] Bligh, E.G., Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 (8), 911–917.
[10] Bräsen, C., Esser, D., Rauch, B., Siebers, B. (2014) Carbohydrate metabolism in *Archaea*: current insights into unusual enzymes and pathways and their regulation. Microbiol. Mol. Biol. Rev. 78 (1), 89–175.
[11] Brettin, T., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Olsen, G.J., Olson, R., Overbeek, R., Parrello, B., Pusch, G.D., Shukla, M., Thomason, J.A., 3rd, Stevens, R., Vonstein, V., Wattam, A.R., Xia, F. (2015) RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci. Rep. 5, 8365, http://dx.doi.org/10.1038/srep08365.
[12] Bright, M., Espada-Hinojosa, S., Lagkouvardos, I., Volland, J.-M. (2014) The giant ciliate *Zoothamnium niveum* and its thiotrophic epibiont *Candidatus* Thiobios zoothamnicoli: a model system to study interspecies cooperation. Front. Microbiol. 5, 145, http://dx.doi.org/10.3389/fmicb.2014.00145.
[13] Burggraf, S., Larsen, N., Woese, C.R., Stetter, K.O. (1993) An intron within the 16S ribosomal RNA gene of the archaeon *Pyrobaculum aerophilum*. Proc. Natl. Acad. Sci. U. S. A. 90 (6), 2547–2550.
[14] Casanueva, A., Galada, N., Baker, G.C., Grant, W.D., Heaphy, S., Jones, B., Yanhe, M., Ventosa, A., Blamey, J., Cowan, D.A. (2008) Nanoarchaeal 16S rRNA gene sequences are widely dispersed in hyperthermophilic and mesophilic halophilic environments. Extremophiles 12 (5), 651–656.
[15] Chernyh, N.A., Mardanov, A.V., Gumerov, V.M., Miroshnichenko, M.L., Lebedinsky, A.V., Merkel, A.Y., Crowe, D., Pimenov, N.V., Rusanov, I.I., Ravin, N.V., Moran, M.A., Bonch-Osmolovskaya, E.A. (2015) Microbial life in Bourlyashchy, the hottest thermal pool of Uzon Caldera, Kamchatka. Extremophiles 19 (6), 1157–1171.
[16] Clingenpeel, S., Kan, J., Macur, R.E., Woyke, T., Lovalvo, D., Varley, J., Inskeep, W.P., Nealson, K., McDermott, T.R. (2013) Yellowstone lake *Nanoarchaeota*. Front. Microbiol. 4, 274, http://dx.doi.org/10.3389/fmicb.2013.00274.
[17] Collingro, A., Köstlbacher, S., Mussmann, M., Stepanauskas, R., Hallam, S.J., Horn, M. (2017) Unexpected genomic features in widespread intracellular bacteria: evidence for motility of marine *Chlamydiae*. ISME J. 11 (10), 2334–2344.
[18] Danczak, R.E., Johnston, M.D., Kenah, C., Slattery, M., Wrighton, K.C., Wilkins, M.J. (2017) Members of the candidate phyla radiation are functionally differentiated by carbon- and nitrogen-cycling capabilities. Microbiome 5 (1), 112, http://dx.doi.org/10.1186/s40168-017-0331-1.
[19] Darling, A.E., Jospin, G., Lowe, E., Matsen, F.A., 4th, Bik, H.M., Eisen, J.A. (2014) PhyloSift: phylogenetic analysis of genomes and metagenomes. PeerJ 2, e243, http://dx.doi.org/10.7717/peerj.243.
[20] Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32 (5), 1792–1797.
[21] Elkins, J.G., Podar, M., Graham, D.E., Makarova, K.S., Wolf, Y., Randau, L., Hedlund, B.P., Brochier-Armanet, C., Kunin, V., Anderson, I., Lapidus, A., Goltsman, E., Barry, K., Koonin, E.V., Hugenholtz, P., Kyrpides, N., Wanner, G., Richardson, P., Keller, M., Stetter, K.O. (2008) A korarchaeal genome reveals insights into the evolution of the Archaea. Proc. Natl. Acad. Sci. U. S. A. 105 (23), 8102–8107.
[22] Elling, F.J., Becker, K.W., Könneke, M., Schröder, J.M., Kellermann, M.Y., Thomm, M., Hinrichs, K.-U. (2016) Respiratory quinones in *Archaea*: phylogenetic distribution and application as biomarkers in the marine environment. Environ. Microbiol. 18 (2), 692–707.
[23] Filker, S., Kaiser, M., Rosselló-Móra, R., Dunthorn, M., Lax, G., Stoeck, T. (2014) "*Candidatus* Haloectosymbiotes riaformosensis" (*Halobacteriaceae*), an archaeal ectosymbiont of the hypersaline ciliate *Platynematum salinarum*. Syst. Appl. Microbiol. 37 (4), 244–251.
[24] Flores, G.E., Campbell, J.H., Kirshtein, J.D., Meneghin, J., Podar, M., Steinberg, J.I., Seewald, J.S., Tivey, M.K., Voytek, M.A., Yang, Z.K., Reysenbach, A.-L. (2011) Microbial community structure of hydrothermal deposits from geochemically different vent fields along the Mid-Atlantic Ridge. Environ. Microbiol. 13 (8), 2158–2171.
[25] Flores, G.E., Hunter, R.C., Liu, Y., Mets, A., Schouten, S., Reysenbach, A.-L. (2012) *Hippea jasoniae* sp. nov. and *Hippea alviniae* sp. nov., thermoacidophilic members of the class *Deltaproteobacteria* isolated from deep-sea hydrothermal vent deposits. Int. J. Syst. Evol. Microbiol. 62 (Pt 6), 1252–1258.

[26] Flores, G.E., Shakya, M., Meneghin, J., Yang, Z.K., Seewald, J.S., Geoff Wheat, C., Podar, M., Reysenbach, A.-L. (2012) Inter-field variability in the microbial communities of hydrothermal vent deposits from a back-arc basin. Geobiology 10 (4), 333–346.

[27] Fujishima, K., Sugahara, J., Tomita, M., Kanai, A. (2010) Large-scale tRNA intron transposition in the archaeal order *Thermoproteales* represents a novel mechanism of intron gain. Mol. Biol. Evol. 27 (10), 2233–2243.

[28] Fukushi, H., Hirai, K. (1992) Proposal of *Chlamydia pecorum* sp. nov. for *Chlamydia* strains derived from ruminants. Int. J. Syst. Bacteriol. 42 (2), 306–308.

[29] Göker, M., Held, B., Lapidus, A., Nolan, M., Spring, S., Yasawong, M., Lucas, S., Glavina Del Rio, T., Tice, H., Cheng, J.-F., Goodwin, L., Tapia, R., Pitluck, S., Liolios, K., Ivanova, N., Mavromatis, K., Mikhailova, N., Pati, A., Chen, A., Palaniappan, K., Brambilla, E., Land, M., Hauser, L., Chang, Y.-J., Jeffries, C.D., Brettin, T., Detter, J.C., Han, C., Rohde, M., Sikorski, J., Woyke, T., Bristow, J., Eisen, J.A., Markowitz, V., Hugenholtz, P., Kyrpides, N.C., Klenk, H.-P. (2010) Complete genome sequence of *Ignisphaera aggregans* type strain (AQ1. S1^{T}). Stand. Genom. Sci. 3 (1), 66–75.

[30] Grayston, J.T., Kuo, C.-C., Campbell, L.A., Wang, S.-P. (1989) *Chlamydia pneumoniae* sp. nov. for *Chlamydia* sp. strain TWAR. Int. J. Syst. Evol. Microbiol. 39 (1), 88–90.

[31] Greening, C., Biswas, A., Carere, C.R., Jackson, C.J., Taylor, M.C., Stott, M.B., Cook, G.M., Morales, S.E. (2016) Genomic and metagenomic surveys of hydrogenase distribution indicate H_2 is a widely utilised energy source for microbial growth and survival. ISME J. 10 (3), 761–777.

[32] Hamilton-Brehm, S.D., Gibson, R.A., Green, S.J., Hopmans, E.C., Schouten, S., van der Meer, M.T.J., Shields, J.P., Damsté, J.S.S., Elkins, J.G. (2013) *Thermodesulfobacterium geofontis* sp. nov., a hyperthermophilic, sulfate-reducing bacterium isolated from Obsidian Pool, Yellowstone National Park. Extremophiles 17 (2), 251–263.

[33] Heider, J., Ma, K., Adams, M.W. (1995) Purification, characterization, and metabolic function of tungsten-containing aldehyde ferredoxin oxidoreductase from the hyperthermophilic and proteolytic archaeon *Thermococcus* strain ES-1. J. Bacteriol. 177 (16), 4757–4764.

[34] Hohn, M.J., Hedlund, B.P., Huber, H. (2002) Detection of 16S rDNA sequences representing the novel phylum "*Nanoarchaeota*": indication for a world-wide distribution in high temperature biotopes. Syst. Appl. Microbiol. 25, 551–554.

[35] Huber, H., Hohn, M.J., Rachel, R., Fuchs, T., Wimmer, V.C., Stetter, K.O. (2002) A new phylum of *Archaea* represented by a nanosized hyperthermophilic symbiont. Nature 417 (6884), 63–67.

[36] Huber, R., Dyba, D., Huber, H., Burggraf, S., Rachel, R. (1998) Sulfur-inhibited *Thermosphaera aggregans* sp. nov., a new genus of hyperthermophilic archaea isolated after its prediction from environmentally derived 16S rRNA sequences. Int. J. Syst. Evol. Microbiol. 48 (1), 31–38.

[37] Imanaka, H., Yamatsu, A., Fukui, T., Atomi, H., Imanaka, T. (2006) Phosphoenolpyruvate synthase plays an essential role for glycolysis in the modified Embden–Meyerhof pathway in *Thermococcus kodakarensis*. Mol. Microbiol. 61 (4), 898–909.

[38] Itoh, T., Suzuki, K., Nakase, T. (1998) Occurrence of introns in the 16S rRNA genes of members of the genus *Thermoproteus*. Arch. Microbiol. 170 (3), 155–161.

[39] Jarrell, K.F., Stark, M., Nair, D.B., Chong, J.P.J. (2011) Flagella and pili are both necessary for efficient attachment of *Methanococcus maripaludis* to surfaces. FEMS Microbiol. Lett. 319 (1), 44–50.

[40] Jay, Z.J., Inskeep, W.P. (2015) The distribution, diversity, and importance of 16S rRNA gene introns in the order *Thermoproteales*. Biol. Direct 10, 35, http://dx.doi.org/10.1186/s13062-015-0065-6.

[41] Jeon, J.H., Lim, J.K., Kim, M.-S., Yang, T.-J., Lee, S.-H., Bae, S.S., Kim, Y.J., Lee, S.H., Lee, J.-H., Kang, S.G., Lee, H.S. (2015) Characterization of the frhAGB-encoding hydrogenase from a non-methanogenic hyperthermophilic archaeon. Extremophiles 19 (1), 109–118.

[42] Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A. (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12), 1647–1649.

[43] Kelley, J. 2017 Expanding Metabolic Diversity of Two Archaeal Phyla: *Nanoarchaeota* and *Korarchaeota*. M. Sc. Thesis, Biology Department, Portland State University, Portland, OR, USA.

[44] Kim, O.-S., Cho, Y.-J., Lee, K., Yoon, S.-H., Kim, M., Na, H., Park, S.-C., Jeon, Y.S., Lee, J.-H., Yi, H., Won, S., Chun, J. (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int. J. Syst. Evol. Microbiol. 62 (Pt 3), 716–721.

[45] Kjems, J., Leffers, H., Olesen, T., Garrett, R.A. (1989) A unique tRNA intron in the variable loop of the extreme thermophile *Thermofilum pendens* and its possible evolutionary implications. J. Biol. Chem. 264 (30), 17834–17837.

[46] Konstantinidis, K.T., Rosselló-Móra, R. (2015) Classifying the uncultivated microbial majority: a place for metagenomic data in the *Candidatus* proposal. Syst. Appl. Microbiol. 38 (4), 223–230.

[47] Konstantinidis, K.T., Rosselló-Móra, R., Amann, R. (2017) Uncultivated microbes in need of their own taxonomy. ISME J. 11 (11), 2399–2406.

[48] Krueger, F. Trim Galore! Babraham Bioinformatics. http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/. (Accessed 2016).

[49] Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S.J., Marra, M.A. (2009) Circos: an information aesthetic for comparative genomics. Genome Res. 19 (9), 1639–1645.

[50] Lapage, S.P., Lessel, E.F., Skerman, V.B.D., Clark, W.A., Seeliger, H.P.R. 1992 International Code of Nomenclature of *Bacteria*. 1990 Revison, ASM Press, Washington, D.C.

[51] Lebedinsky, A.V., Mardanov, A.V., Kublanov, I.V., Gumerov, V.M., Beletsky, A.V., Perevalova, A.A., Bidzhieva, S.K., Bonch-Osmolovskaya, E.A., Skryabin, K.G., Ravin, N.V. (2014) Analysis of the complete genome of *Fervidococcus fontis* confirms the distinct phylogenetic position of the order *Fervidicoccales* and suggests its environmental function. Extremophiles 18 (2), 295–309.

[52] Lee, H.S., Kang, S.G., Bae, S.S., Lim, J.K., Cho, Y., Kim, Y.J., Jeon, J.H., Cha, S.-S., Kwon, K.K., Kim, H.-T., Park, C.-J., Lee, H.-W., Kim, S.I., Chun, J., Colwell, R.R., Kim, S.-J., Lee, J.-H. (2008) The complete genome sequence of *Thermococcus onnurineus* NA1 reveals a mixed heterotrophic and carboxydotrophic metabolism. J. Bacteriol. 190 (22), 7491–7499.

[53] Lee, S.H., Kim, M.-S., Kim, Y.J., Kim, T.W., Kang, S.G., Lee, H.S. (2017) Transcriptomic profiling and its implications for the H_2 production of a non-methanogen deficient in the frhAGB-encoding hydrogenase. Appl. Microbiol. Biotechnol. 101 (12), 5081–5088.

[54] Letunic, I., Bork, P. (2016) Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res. 44 (W1), W242–W245, http://dx.doi.org/10.1093/nar/gkw290.

[55] Li, Y., Altman, S. (2004) In search of RNase P RNA from microbial genomes. RNA 10 (10), 1533–1540.

[56] Lowe, T.M., Chan, P.P. (2016) tRNAscan-SE on-line: integrating search and context for analysis of transfer RNA genes. Nucleic Acids Res. 44 (W1), W54–W57, http://dx.doi.org/10.1093/nar/gkw413.

[57] Lykke-Andersen, J., Aagaard, C., Semionenkov, M., Garrett, R.A. (1997) Archaeal introns: splicing, intercellular mobility and evolution. Trends Biochem. Sci. 22 (9), 326–331.

[58] Mai, X., Adams, M.W. (1996) Purification and characterization of two reversible and ADP-dependent acetyl coenzyme A synthetases from the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. 178 (20), 5897–5903.

[59] Makarova, K.S., Sorokin, A.V., Novichkov, P.S., Wolf, Y.I., Koonin, E.V. (2007) Clusters of orthologous genes for 41 archaeal genomes and implications for evolutionary genomics of *Archaea*. Biol. Direct 2, 33, http://dx.doi.org/10.1186/1745-6150-2-33.

[60] Makarova, K.S., Wolf, Y.I., Koonin, E.V. (2015) Archaeal clusters of orthologous genes (arCOGs): an update and application for analysis of shared features between *Thermococcales*, *Methanococcales*, and *Methanobacteriales*. Life 5 (1), 818–840.

[61] Ma, K., Hutchins, A., Sung, S.J., Adams, M.W. (1997) Pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon, *Pyrococcus furiosus*, functions as a CoA-dependent pyruvate decarboxylase. Proc. Natl. Acad. Sci. U. S. A. 94 (18), 9608–9613.

[62] Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C.J., Lu, S., Chitsaz, F., Derbyshire, M.K., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Geer, L.Y., Bryant, S.H. (2017) CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic Acids Res. 45 (D1), D200–D203, http://dx.doi.org/10.1093/nar/gkw1129.

[63] McCliment, E.A., Voglesonger, K.M., O'Day, P.A., Dunn, E.E., Holloway, J.R., Cary, S.C. (2006) Colonization of nascent, deep-sea hydrothermal vents by a novel archaeal and nanoarchaeal assemblage. Environ. Microbiol. 8 (1), 114–125.

[64] McOrist, S., Gebhart, C.J., Boid, R., Barns, S.M. (1995) Characterization of *Lawsonia intracellularis* gen. nov., sp. nov., the obligately intracellular bacterium of porcine proliferative enteropathy. Int. J. Syst. Bacteriol. 45 (4), 820–825.

[65] Meador, T.B., Gagen, E.J., Loscar, M.E., Goldhammer, T., Yoshinaga, M.Y., Wendt, J., Thomm, M., Hinrichs, K.-U. (2014) *Thermococcus kodakarensis* modulates its polar membrane lipids and elemental composition according to growth stage and phosphate availability. Front. Microbiol. 5, 10, http://dx.doi.org/10.3389/fmicb.2014.00010.

[66] Mediannikov, O., Sekeyová, Z., Birg, M.-L., Raoult, D. (2010) A novel obligate intracellular gamma-proteobacterium associated with ixodid ticks, *Diplorickettsia massiliensis*, gen. nov., sp. nov. PLoS One 5 (7), e11478, http://dx.doi.org/10.1371/journal.pone.0011478.

[67] Menzel, P., Gudbergsdóttir, S.R., Rike, A.G., Lin, L., Zhang, Q., Contursi, P., Moracci, M., Kristjansson, J.K., Bolduc, B., Gavrilov, S., Ravin, N., Mardanov, A., Bonch-Osmolovskaya, E., Young, M., Krogh, A., Peng, X. (2015) Comparative metagenomics of eight geographically remote terrestrial hot springs. Microb. Ecol. 70 (2), 411–424.

[68] Munson-McGee, J.H., Field, E.K., Bateson, M., Rooney, C., Stepanauskas, R., Young, M.J. (2015) *Nanoarchaeota*, their *Sulfolobales* host, and *Nanoarchaeota* virus distribution across Yellowstone National Park hot springs. Appl. Environ. Microbiol. 81 (22), 7860–7868.

[69] Nakagawa, T., Ishibashi, J.-I., Maruyama, A., Yamanaka, T., Morimoto, Y., Kimura, H., Urabe, T., Fukui, M. (2004) Analysis of dissimilatory sulfite reductase and 16S rRNA gene fragments from deep-sea hydrothermal sites of the Suiyo Seamount, Izu-Bonin Arc, Western Pacific. Appl. Environ. Microbiol. 70 (1), 393–403.

[70] Nawrocki, E.P., Burge, S.W., Bateman, A., Daub, J., Eberhardt, R.Y., Eddy, S.R., Floden, E.W., Gardner, P.P., Jones, T.A., Tate, J., Finn, R.D. (2015) Rfam 12.0: updates to the RNA families database. Nucleic Acids Res. 43 (Database issue), D130–D137, http://dx.doi.org/10.1093/nar/gku1063.

[71] Nawrocki, E.P., Eddy, S.R. (2013) Infernal 1.1: 100-fold faster RNA homology searches. Bioinformatics 29 (22), 2933–2935.

[72] NCBI Resource Coordinators, (2017) Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 45 (D1), D12–D17, http://dx.doi.org/10.1093/nar/gkw1071.
[73] Niederberger, T.D., Götz, D.K., McDonald, I.R., Ronimus, R.S., Morgan, H.W. (2006) *Ignisphaera aggregans* gen. nov., sp. nov., a novel hyperthermophilic crenarchaeote isolated from hot springs in Rotorua and Tokaanu, New Zealand. Int. J. Syst. Evol. Microbiol. 56 (Pt 5), 965–971.
[74] Nishihara, M., Koga, Y. (1987) Extraction and composition of polar lipids from the archaebacterium, *Methanobacterium thermoautotrophicum*: effective extraction of tetraether lipids by an acidified solvent. J. Biochem. 101 (4), 997–1005.
[75] Nomura, N., Sako, Y., Uchida, A. (1998) Molecular characterization and postsplicing fate of three introns within the single rRNA operon of the hyperthermophilic archaeon *Aeropyrum pernix* K1. J. Bacteriol. 180 (14), 3635–3643.
[76] Oger, P., Sokolova, T.G., Kozhevnikova, D.A., Chernyh, N.A., Bartlett, D.H., Bonch-Osmolovskaya, E.A., Lebedinsky, A.V. (2011) Complete genome sequence of the hyperthermophilic archaeon *Thermococcus* sp. strain AM4, capable of organotrophic growth and growth at the expense of hydrogenogenic or sulfidogenic oxidation of carbon monoxide. J. Bacteriol. 193 (24), 7019–7020.
[77] Overbeek, R., Olson, R., Pusch, G.D., Olsen, G.J., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A.R., Xia, F., Stevens, R. (2014) The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). Nucleic Acids Res. 42 (Database issue), D206–D214, http://dx.doi.org/10.1093/nar/gkt1226.
[78] Paper, W., Jahn, U., Hohn, M.J., Kronner, M., Näther, D.J., Burghardt, T., Rachel, R., Stetter, K.O., Huber, H. (2007) *Ignicoccus hospitalis* sp. nov., the host of "*Nanoarchaeum equitans*". Int. J. Syst. Evol. Microbiol. 57 (Pt 4), 803–808.
[79] Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W. (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25 (7), 1043–1055.
[80] Peng, Y., Leung, H.C.M., Yiu, S.M., Chin, F.Y.L. (2012) IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. Bioinformatics 28 (11), 1420–1428.
[81] Peng, Y., Leung, H.C.M., Yiu, S.-M., Chin, F.Y.L. (2010) IDBA—a practical iterative de Bruijn graph de novo assemblerL. In: Annual International Conference on Research in Computational Molecular Biology, Springer, pp. 426–440.
[82] Pisa, K.Y., Huber, H., Thomm, M., Müller, V. (2007) A sodium ion-dependent A1AO ATP synthase from the hyperthermophilic archaeon *Pyrococcus furiosus*. FEBS J. 274 (15), 3928–3938.
[83] Podar, M., Makarova, K.S., Graham, D.E., Wolf, Y.I., Koonin, E.V., Reysenbach, A.-L. (2013) Insights into archaeal evolution and symbiosis from the genomes of a nanoarchaeon and its inferred crenarchaeal host from Obsidian Pool, Yellowstone National Park. Biol. Direct 8, 9, http://dx.doi.org/10.1186/1745-6150-8-9.
[84] Rachel, R., Wyschkony, I., Riehl, S., Huber, H. (2002) The ultrastructure of *Ignicoccus*: evidence for a novel outer membrane and for intracellular vesicle budding in an archaeon. Archaea 1 (1), 9–18.
[85] Randau, L., Münch, R., Hohn, M.J., Jahn, D., Söll, D. (2005) *Nanoarchaeum equitans* creates functional tRNAs from separate genes for their 5′-and 3′-halves. Nature 433 (7025), 537–541.
[86] Randau, L., Pearson, M., Söll, D. (2005) The complete set of tRNA species in *Nanoarchaeum equitans*. FEBS Lett. 579 (13), 2945–2947.
[87] Randau, L., Schröder, I., Söll, D. (2008) Life without RNase P. Nature 453 (7191), 120–123.
[88] Ravin, N.V., Mardanov, A.V., Beletsky, A.V., Kublanov, I.V., Kolganova, T.V., Lebedinsky, A.V., Chernyh, N.A., Bonch-Osmolovskaya, E.A., Skryabin, K.G. (2009) Complete genome sequence of the anaerobic, protein-degrading hyperthermophilic crenarchaeon *Desulfurococcus kamchatkensis*. J. Bacteriol. 191 (7), 2371–2379.
[89] Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N.N., Anderson, I.J., Cheng, J.-F., Darling, A., Malfatti, S., Swan, B.K., Gies, E.A., Dodsworth, J.A., Hedlund, B.P., Tsiamis, G., Sievert, S.M., Liu, W.-T., Eisen, J.A., Hallam, S.J., Kyrpides, N.C., Stepanauskas, R., Rubin, E.M., Hugenholtz, P., Woyke, T. (2013) Insights into the phylogeny and coding potential of microbial dark matter. Nature 499 (7459), 431–437.
[90] Rodriguez-R, L.M., Konstantinidis, K.T. (2016) The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. PeerJ Prepr., e1900v, http://dx.doi.org/10.7287/peerj.preprints.1900v1.
[91] Sato, T., Atomi, H., Imanaka, T. (2007) Archaeal type III RuBisCOs function in a pathway for AMP metabolism. Science 315 (5814), 1003–1006.
[92] Schlegel, K., Leone, V., Faraldo-Gómez, J.D., Müller, V. (2012) Promiscuous archaeal ATP synthase concurrently coupled to Na^+ and H^+ translocation. Proc. Natl. Acad. Sci. U. S. A. 109 (3), 947–952.
[93] Schopf, S., Wanner, G., Rachel, R., Wirth, R. (2008) An archaeal bi-species biofilm formed by *Pyrococcus furiosus* and *Methanopyrus kandleri*. Arch. Microbiol. 190 (3), 371–377.
[94] Schouten, S., van der Meer, M.T.J., Hopmans, E.C., Rijpstra, W.I.C., Reysenbach, A.-L., Ward, D.M., Sinninghe Damsté, J.S. (2007) Archaeal and bacterial glycerol dialkyl glycerol tetraether lipids in hot springs of Yellowstone National Park. Appl. Environ. Microbiol. 73 (19), 6181–6191.
[95] Schuster, L., Bright, M. (2016) A novel colonial ciliate *Zoothamnium ignavum* sp. nov. (*Ciliophora*, *Oligohymenophorea*) and its ectosymbiont *Candidatus* Navis piranensis gen. nov., sp. nov. from shallow-water wood falls. PLoS One 11 (9), e0162834, http://dx.doi.org/10.1371/journal.pone.0162834.
[96] Sokolova, T.G., Henstra, A.-M., Sipma, J., Parshina, S.N., Stams, A.J.M., Lebedinsky, A.V. (2009) Diversity and ecophysiological features of thermophilic carboxydotrophic anaerobes. FEMS Microbiol. Ecol. 68 (2), 131–141.
[97] Sokolova, T.G., Jeanthon, C., Kostrikina, N.A., Chernyh, N.A., Lebedinsky, A.V., Stackebrandt, E., Bonch-Osmolovskaya, E.A. (2004) The first evidence of anaerobic CO oxidation coupled with H_2 production by a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Extremophiles 8 (4), 317–323.
[98] Søndergaard, D., Pedersen, C.N.S., Greening, C. (2016) HydDB: a web tool for hydrogenase classification and analysis. Sci. Rep. 6, 34212, http://dx.doi.org/10.1038/srep34212.
[99] Spang, A., Saw, J.H., Jørgensen, S.L., Zaremba-Niedzwiedzka, K., Martijn, J., Lind, A.E., van Eijk, R., Schleper, C., Guy, L., Ettema, T.J.G. (2015) Complex *Archaea* that bridge the gap between prokaryotes and eukaryotes. Nature 521 (7551), 173–179.
[100] Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30 (9), 1312–1313.
[101] Sugahara, J., Kikuta, K., Fujishima, K., Yachie, N., Tomita, M., Kanai, A. (2008) Comprehensive analysis of archaeal tRNA genes reveals rapid increase of tRNA introns in the order *Thermoproteales*. Mol. Biol. Evol. 25 (12), 2709–2716.
[102] Takai, K., Horikoshi, K. (1999) Molecular phylogenetic analysis of archaeal intron-containing genes coding for rRNA obtained from a deep-subsurface geothermal water pool. Appl. Environ. Microbiol. 65 (12), 5586–5589.
[103] Ultsch, A., Mörchen, F. 2005 ESOM-Maps: Tools for Clustering, Visualization, and Classification With Emergent SOM, Technical Report Dept. of Mathematics and Computer Science, University of Marburg, Germany, no. 46.
[104] Vannier, P., Marteinsson, V.T., Fridjonsson, O.H., Oger, P., Jebbar, M. (2011) Complete genome sequence of the hyperthermophilic, piezophilic, heterotrophic, and carboxydotrophic archaeon *Thermococcus barophilus* MP. J. Bacteriol. 193 (6), 1481–1482.
[105] Waters, E., Hohn, M.J., Ahel, I., Graham, D.E., Adams, M.D., Barnstead, M., Beeson, K.Y., Bibbs, L., Bolanos, R., Keller, M., Kretz, K., Lin, X., Mathur, E., Ni, J., Podar, M., Richardson, T., Sutton, G.G., Simon, M., Soll, D., Stetter, K.O., Short, J.M., Noordewier, M. (2003) The genome of *Nanoarchaeum equitans*: insights into early archaeal evolution and derived parasitism. Proc. Natl. Acad. Sci. U. S. A. 100 (22), 12984–12988.
[106] Wurch, L., Giannone, R.J., Belisle, B.S., Swift, C., Utturkar, S., Hettich, R.L., Reysenbach, A.-L., Podar, M. (2016) Genomics-informed isolation and characterization of a symbiotic *Nanoarchaeota* system from a terrestrial geothermal environment. Nat. Commun. 7, 12115, http://dx.doi.org/10.1038/ncomms12115.
[107] Wu, Y.-W., Tang, Y.-H., Tringe, S.G., Simmons, B.A., Singer, S.W. (2014) MaxBin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. Microbiome 2, 26, http://dx.doi.org/10.1186/2049-2618-2-26.
[108] Yoon, S.-H., Ha, S.-M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J. (2016) Introducing EzBioCloud: a taxonomically united database of 16S rRNA and whole genome assemblies. Int. J. Syst. Evol. Microbiol. 67 (5), 1613–1617.
[109] Yoshinaga, M.Y., Kellermann, M.Y., Rossel, P.E., Schubotz, F., Lipp, J.S., Hinrichs, K.-U. (2011) Systematic fragmentation patterns of archaeal intact polar lipids by high-performance liquid chromatography/electrospray ionization ion-trap mass spectrometry. Rapid Commun. Mass Spectrom. 25 (23), 3563–3574.
[110] Zolghadr, B., Klingl, A., Koerdt, A., Driessen, A.J.M., Rachel, R., Albers, S.-V. (2010) Appendage-mediated surface adherence of *Sulfolobus solfataricus*. J. Bacteriol. 192 (1), 104–110.
[111] Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31 (13), 3406–3415.